Supplementary Information

Computational design of Small Transcription Activating RNAs (STARs) for versatile and dynamic gene regulation

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Supplementary Table 1. Plasmids used in this study. Abbreviations are as follows: Promoter 1-5 = constitutive promoter variants (see Supplementary Table 3), BBa_J23119 = strong constitutive promoter, RBS 1-5 = ribosome binding site variants (see Supplementary Table 3), BBa_B0034* and RBS_RFP = ribosome binding sites used for dCas9 and mRFP, ptet = TetR repressible promoter, plux (BBa_R0062)* = LuxR inducible promoter, sfGFP = SuperFolder green fluorescent protein[1], GFPmut3b-ASV = green fluorescent protein with ASV degradation tag[2], mRFP = red fluorescent protein, YFP (BBa_E0030)* = yellow fluorescent protein, catechol (BBa_K316003)* = catechol (2,3)-dioxygenase, LuxR (BBa_C0062)* = AHL inducible transcription factor, CheZ = CheZ chemotaxis regulator, tetR = tet repressor protein, vioA-E = genes from deoxyviolacein metabolic pathway, dCas9 = catalytically dead Cas9, Csy4hp = Csy4 RNA hairpin[3], sgRNA = single guide RNA, TrrnB = rrnB terminator, BBa_B0015* = B0015 terminator, T500 = T500 terminator, CmR = chloramphenicol resistance cassette, AmpR = ampicillin resistance cassette, SpecR = spectinomycin resistance cassette, p15A = p15A origin of replication, ColE1 = ColE1 origin of replication, CDF = CDF origin of replication and R6K = R6K origin of replication. Several of the DNA sequences (indicted by *) were obtained from the iGEM Registry of Standard Biological Parts (parts.igem.org), BioBrick numbers (BBa) indicated in name or brackets. We would like to acknowledge that the dCas9 expression plasmid[4] (indicated in table below with ‡) was a gift from Stanley Qi's laboratory, Stanford University.

Plasmid #	Plasmid architecture	Name	Figure
N/A	Promoter 1 – Target – RBS 1 – sfGFP – TrrnB – CmR – p15A origin	Target sfGFP	Na
N/A	Promoter 1 – STAR– t500 – ColE1 origin – AmpR	STAR	Na
pJBL6063	Promoter 1 – Target 1 – RBS 1 – GFPmut3b-ASV – TrrnB – CmR – p15A origin	Target Variant 1 GFPmut3b-ASV	Na
pJBL6064	Promoter 1 – toehold switch 1 – linker – GFPmut3b-ASV – TrrnB – CmR – p15A origin	Toehold switch 1 GFPmut3b-ASV	SI 6
pJBL3869	Promoter 1 – toehold switch 1 – linker – sfGFP – TrrnB – CmR – p15A origin	Toehold switch 1 sfGFP	SI 6
pJBL3870	Promoter 1 – stability hairpin – toehold trigger 1 – t500 – ColE1 origin – AmpR	Toehold trigger 1	SI 6
pJB6075	Promoter 1 – stability hairpin – RBS 1 – sfGFP – TrrnB – CmR – p15A origin	Promoter 1-RBS 1	SI 12

pJB6076	Promoter 2 – stability hairpin – RBS 1 – sfGFP – TrrnB – CmR – p15A origin	Promoter 2-RBS 1	SI 12
pJB6077	Promoter 3 – stability hairpin – RBS 1 – sfGFP – TrrnB – CmR – p15A origin	Promoter 3-RBS 1	SI 12
pJB6078	Promoter 4 – stability hairpin – RBS 1 – sfGFP – TrrnB – CmR – p15A origin	Promoter 4-RBS 1	SI 12
pJB6079	Promoter 5 – stability hairpin – RBS 1 – sfGFP – TrrnB – CmR – p15A origin	Promoter 5-RBS 1	SI 12
pJB6080	Promoter 1 – stability hairpin – RBS 2 – sfGFP – TrrnB – CmR – p15A origin	Promoter 1-RBS 2	SI 12
pJB6081	Promoter 1 – stability hairpin – RBS 3 – sfGFP – TrrnB – CmR – p15A origin	Promoter 1-RBS 3	SI 12
pJB6082	Promoter 1 – stability hairpin – RBS 4 – sfGFP – TrrnB – CmR – p15A origin	Promoter 1-RBS 4	SI 12
pJB6083	Promoter 1 – stability hairpin – RBS 5 – sfGFP – TrrnB – CmR – p15A origin	Promoter 1-RBS 5	SI 12
pJBL4970	Promoter 1 – Target 5 – RBS 1 – sfGFP – TrrnB – CmR – p15A origin	RBS 1 /Promoter 1 Variant	2a, 3a, 5a, SI 13, SI 14
pJBL5919	Promoter 1 – Target 5 – RBS 2 – sfGFP – TrrnB – CmR – p15A origin	RBS 2 /Promoter 1 Variant	2a, SI 13, SI 14
pJBL5921	Promoter 1 – Target 5 – RBS 3 – sfGFP – TrrnB – CmR – p15A origin	RBS 3 /Promoter 1 Variant	2a, SI 13, SI 14
pJBL5922	Promoter 1 – Target 5 – RBS 4 – sfGFP – TrrnB – CmR – p15A origin	RBS 4 /Promoter 1 Variant	2a, SI 13, SI 14
pJBL5924	Promoter 1 – Target 5 – RBS 5 – sfGFP – TrrnB – CmR – p15A origin	RBS 5 /Promoter 1 Variant	2a, SI 13, SI 14
pJBL5937	Promoter 2 – Target 5 – RBS 1 – sfGFP – TrrnB – CmR – p15A origin	RBS 1 /Promoter 2 Variant	2a, SI 13
pJBL5981	Promoter 2 – Target 5 – RBS 2 – sfGFP – TrrnB – CmR – p15A origin	RBS 2 /Promoter 2 Variant	2a, SI 13
pJBL5982	Promoter 2 – Target 5 – RBS 3 – sfGFP – TrrnB – CmR – p15A origin	RBS 3 /Promoter 2 Variant	2a, SI 13
pJBL5983	Promoter 2 – Target 5 – RBS 4 – sfGFP – TrrnB – CmR – p15A origin	RBS 4 /Promoter 2 Variant	2a, SI 13
pJBL5984	Promoter 2 – Target 5 – RBS 5 – sfGFP – TrrnB – CmR – p15A origin	RBS 5 /Promoter 2 Variant	2a, SI 13

pJBL5935	Promoter 3 – Target 5 – RBS 1 – sfGFP – TrrnB – CmR – p15A origin	RBS 1 /Promoter 3 Variant	2a, SI 13
pJBL5989	Promoter 3 – Target 5 – RBS 2 – sfGFP – TrrnB – CmR – p15A origin	RBS 2 /Promoter 3 Variant	2a, SI 13
pJBL5990	Promoter 3 – Target 5 – RBS 3 – sfGFP – TrrnB – CmR – p15A origin	RBS 3 /Promoter 3 Variant	2a, SI 13
pJBL5991	Promoter 3– Target 5 – RBS 4 – sfGFP – TrrnB – CmR – p15A origin	RBS 4 /Promoter 3 Variant	2a, SI 13
pJBL5992	Promoter 3 – Target 5 – RBS 5 – sfGFP – TrrnB – CmR – p15A origin	RBS 5 /Promoter 3 Variant	2a, SI 13
pJBL5934	Promoter 4 – Target 5 – RBS 1 – sfGFP – TrrnB – CmR – p15A origin	RBS 1 /Promoter 4 Variant	2a, SI 13
pJBL5985	Promoter 4 – Target 5 – RBS 2 – sfGFP – TrrnB – CmR – p15A origin	RBS 2 /Promoter 4 Variant	2a, SI 13
pJBL5986	Promoter 4 – Target 5 – RBS 3 – sfGFP – TrrnB – CmR – p15A origin	RBS 3 /Promoter 4 Variant	2a, SI 13
pJBL5987	Promoter 4 – Target 5 – RBS 4 – sfGFP – TrrnB – CmR – p15A origin	RBS 4 /Promoter 4 Variant	2a, SI 13
pJBL5988	Promoter 4 – Target 5 – RBS 5 – sfGFP – TrrnB – CmR – p15A origin	RBS 5 /Promoter 4 Variant	2a, SI 13
pJBL5932	Promoter 5 – Target 5 – RBS 1 – sfGFP – TrrnB – CmR – p15A origin	RBS 1 /Promoter 5 Variant	2a, SI 13
pJBL5993	Promoter 5 – Target 5 – RBS 2 – sfGFP – TrrnB – CmR – p15A origin	RBS 2 /Promoter 5 Variant	2a, SI 13
pJBL5994	Promoter 5 – Target 5 – RBS 3 – sfGFP – TrrnB – CmR – p15A origin	RBS 3 /Promoter 5 Variant	2a, SI 13
pJBL5995	Promoter 5 – Target 5 – RBS 4 – sfGFP – TrrnB – CmR – p15A origin	RBS 4 /Promoter 5 Variant	2a, SI 13
pJBL5996	Promoter 5 – Target 5 – RBS 5 – sfGFP – TrrnB – CmR – p15A origin	RBS 5 /Promoter 5 Variant	2a, SI 13
pJBL4826	ptet – B0034 – luxR – B0015 – plux – STAR 5 – t500 – ColE1 origin – AmpR	AHL Inducible STAR Variant 5	2b, 6c, SI 22
pJBL5945	ptet – B0034 – luxR – B0015 – plux – STAR 8 – t500 – ColE1 origin – AmpR	AHL Inducible STAR Variant 8	2b
pJBL5946	ptet – B0034 – luxR – B0015 – plux – STAR 6 – t500 – ColE1 origin – AmpR	AHL Inducible	2b

		STAR Variant 6	
pJBL4882	Promoter 1 – Target 5 – RBS_RFP – mRFP – TrrnB – CmR – p15A origin	Target Variant 5 mRFP	3a, 5a
pJBL5938	Promoter 1– Target 8 – RBS_RFP – mRFP – TrrnB – CmR – p15A origin	Target Variant 8 mRFP	3a
pJBL5939	Promoter 1– Target 6 – RBS_RFP – mRFP – TrrnB – CmR – p15A origin	Target Variant 6 mRFP	3a
pJBL4880	Promoter 1– Target 5 – B0034 – YFP – TrrnB – CmR – p15A origin	Target Variant 5 YFP	3a
pJBL5940	Promoter 1– Target 6 – B0034 – YFP – TrrnB – CmR – p15A origin	Target Variant 8 YFP	3a
pJBL5941	Promoter 1– Target 8 – B0034 – YFP – TrrnB – CmR – p15A origin	Target Variant 6 YFP	3a
pJBL3877	Promoter 1 – Target 5 – RBS 1 – Catechol – TrrnB – CmR – p15A origin	Target Variant 5 Catechol	3c
pJBL6084	Promoter 3 – Target 5 – RBS 1 – vioA – vioB – vioC – vioE – SpecR – R6K	Target Variant 5 vioABCE	4b
pJBL6060	Promoter 1 – Target 5 – RBS 1 – Catechol – TrrnB – SpecR – CDF origin	Target Variant 5 Catechol	5a, SI 18
pJBL6061	Promoter 1 – Target 5 – RBS 1 – sfGFP – TrrnB – Promoter 1 – Target 5 – RBS_RFP – mRFP – TrrnB – CmR – p15A origin	Target Variant 5 sfGFP and mRFP	5a, SI 18
pJBL6062	Promoter 1 – Target 10 – Csy4hp – STAR 50 – t500 – tetR – ptet – csy4 – SpecR – CDF origin	Stage 2 activation-activation cascade	5b
pJBL5576	Promoter 1 – Input B– t500 – ColE1 origin – AmpR	Input B AND Gate (From STAR Variant 1)	5d
pJBL5577	Promoter 1 – Input A– t500 – ColE1 origin – AmpR	Input A AND Gate (From STAR Variant 1)	5d
pJBL5536	Promoter 1 – Input B– t500 – Promoter 1 – Input A– t500 – ColE1 origin – AmpR	Input AB AND Gate	5d
‡pdCas9-bacteria pJBL622	TetR – ptet – B0034 – dCas9 – B0015– p15A - CmR	dCas9	6a, 6b, SI 20
pJBL5564	Promoter 3 – Target 5 – Csy4hp – sgRNA – TrrnB – SpecR – CDF origin	Target Variant 5 sgRNA	6a, SI 20
pJBL5959	Promoter 1 – STAR– t500 – ColE1 origin – AmpR – BBa_J23119 – RBS_RFP – mRFP – B0015	STAR mRFP	6a, SI 20

pJBL5998	Promoter 1 – TrrnB – ColE1 origin – AmpR – BBa_J23119 – RBS_RFP – mRFP – B0015	No-STAR mRFP Control	6a, SI 20
pJBL5504	Promoter 3 – sgRNA – TrrnB – SpecR – CDF origin	Constitutive sgRNA	6a, SI 20
pJBL5506	Promoter 3 – sgRNA – TrrnB – SpecR – CDF origin – BBa_J23119 – Target5 – RBS_RFP – mRFP – TrrnB	Constitutive sgRNA Target Variant 5 mRFP	6b
pJBL5502	TetR – Promoter 3 – Target 5 – B0034 – dCas9 – B0015 – p15A - CmR	Target Variant 5 dCas9	6c, SI 22
pJBL5568	Promoter 3 – Target 5 – Csy4hp – sgRNA – TrrnB – SpecR – CDF origin – Promoter 1 – Target 5 – RBS_RFP – mRFP – TrrnB	FFL Target Variant 5 sgRNA and mRFP	6c, SI 22
pJBL5999	Promoter 1 –TrrnB – SpecR – CDF origin	No sgRNA control	Control
pJBL001	TrrnB – CmR – p15A origin	No-Target control	Control
pJBL002	Promoter 1 – TrrnB – ColE1 origin – AmpR	No-STAR control	Control

Supplementary Table 2. Examples of DNA plasmid sequences. Abbreviations as described in Supplementary Table 1.

Name	Sequence (5' to 3')
Example Target (Variant 1) DNA Plasmid: (Promoter 1-Target 1-RBS 1-sfGFP-TrrnB-CmR-p15A origin)	GAATTCTAAAGATCTTTGACAGCTAGCTCAGTCCTAGGTATAATACTAGTCCATCTTACCTTTG CATCTCTATCGTTCTCATCTCATCCTGCGGGGAATGTATACAGTTCATGTATATATTCCCCGC TTTTTTTTTGGATCTAGGAGGAAGGATCTATGAGCAAAGGAGAAGAACTTTTCACTGGAGTTG TCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCCGTGGAGAGG GTGAAGGTGATGCTACAAACGGAAAACTCACCCTTAAATTTATTTGCACTACTGGAAAACTAC CTGTTCCGTGGCCAACACTTGTCACTACTCTGACCTATGGTGTTCAATGCTTTTCCCGTTATC CGGATCACATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAA CGCACTATATCTTTCAAAGATGACGGGACCTACAAGACGCGTGCTGAAGTCAAGTTTGAAGG TGATACCCTTGTTAATCGTATCGAGTTAAAGGGTATTGATTTTAAAGAAGATGGAAACATTCTT GGACACAAACTCGAGTACAACTTTAACTCACACAATGTATACATCACGGCAGACAAACAAAAG AATGGAATCAAAGCTAACTTCAAAATTCGCCACAACGTTGAAGATGGTTCCGTTCAACTAGCA GACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTAC CTGTCGACACAATCTGTCCTTTCGAAAGATCCCAACGAAAAGCGTGACCACATGGTCCTTCTT GAGTTTGTAACTGCTGCTGGGATTACACATGGCATGGATGAGCTCTACAAATAAGGATCTGA AGCTTGGGCCCGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCAT CATCATCATCATTGAGTTTAAACGGTCTCCAGCTTGGCTGTTTTGGCGGATGAGAGAAGATTT TCAGCCTGATACAGATTAAATCAGAACGCAGAAGCGGTCTGATAAAACAGAATTTGCCTGGC GGCAGTAGCGCGGTGGTCCCACCTGACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGCG CCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAAC GAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACTGGATC CTTACTCGAGTCTAGACTGCAGTTGATCGGGCACGTAAGAGGTTCCAACTTTCACCATAATGA AATAAGATCACTACCGGGCGTATTTTTTGAGTTATCGAGATTTTCAGGAGCTAAGGAAGCTAA AATGGAGAAAAAAATCACTGGATATACCACCGTTGATATATCCCAATGGCATCGTAAAGAACA TTTTGAGGCATTTCAGTCAGTTGCTCAATGTACCTATAACCAGACCGTTCAGCTGGATATTAC GGCCTTTTTAAAGACCGTAAAGAAAAATAAGCACAAGTTTTATCCGGCCTTTATTCACATTCTT GCCCGCCTGATGAATGCTCATCCGGAATTTCGTATGGCAATGAAAGACGGTGAGCTGGTGAT ATGGGATAGTGTTCACCCTTGTTACACCGTTTTCCATGAGCAAACTGAAACGTTTTCATCGCT CTGGAGTGAATACCACGACGATTTCCGGCAGTTTCTACACATATATTCGCAAGATGTGGCGT GTTACGGTGAAAACCTGGCCTATTTCCCTAAAGGGTTTATTGAGAATATGTTTTTCGTCTCAG CCAATCCCTGGGTGAGTTTCACCAGTTTTGATTTAAACGTGGCCAATATGGACAACTTCTTCG CCCCCGTTTTCACCATGGGCAAATATTATACGCAAGGCGACAAGGTGCTGATGCCGCTGGC GATTCAGGTTCATCATGCCGTTTGTGATGGCTTCCATGTCGGCAGAATGCTTAATGAATTACA

	ACAGTACTGCGATGAGTGGCAGGGCGGGGCGTAATTTGATATCGAGCTCGCTTGGACTCCT GTTGATAGATCCAGTAATGACCTCAGAACTCCATCTGGATTTGTTCAGAACGCTCGGTTGCC GCCGGGCGTTTTTTATTGGTGAGAATCCAAGCCTCCGATCAACGTCTCATTTTCGCCAAAAGT TGGCCCAGGGCTTCCCGGTATCAACAGGGACACCAGGATTTATTTATTCTGCGAAGTGATCT TCCGTCACAGGTATTTATTCGGCGCAAAGTGCGTCGGGTGATGCTGCCAACTTACTGATTTA GTGTATGATGGTGTTTTTGAGGTGCTCCAGTGGCTTCTGTTTCTATCAGCTGTCCCTCCTGTT CAGCTACTGACGGGGTGGTGCGTAACGGCAAAAGCACCGCCGGACATCAGCGCTAGCGGA GTGTATACTGGCTTACTATGTTGGCACTGATGAGGGTGTCAGTGAAGTGCTTCATGTGGCAG GAGAAAAAAGGCTGCACCGGTGCGTCAGCAGAATATGTGATACAGGATATATTCCGCTTCCT CGCTCACTGACTCGCTACGCTCGGTCGTTCGACTGCGGCGAGCGGAAATGGCTTACGAACG GGGCGGAGATTTCCTGGAAGATGCCAGGAAGATACTTAACAGGGAAGTGAGAGGGCCGCG GCAAAGCCGTTTTTCCATAGGCTCCGCCCCCCTGACAAGCATCACGAAATCTGACGCTCAAA TCAGTGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGCGGCTCC CTCGTGCGCTCTCCTGTTCCTGCCTTTCGGTTTACCGGTGTCATTCCGCTGTTATGGCCGCG TTTGTCTCATTCCACGCCTGACACTCAGTTCCGGGTAGGCAGTTCGCTCCAAGCTGGACTGT ATGCACGAACCCCCCGTTCAGTCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTC CAACCCGGAAAGACATGCAAAAGCACCACTGGCAGCAGCCACTGGTAATTGATTTAGAGGAG TTAGTCTTGAAGTCATGCGCCGGTTAAGGCTAAACTGAAAGGACAAGTTTTGGTGACTGCGC TCCTCCAAGCCAGTTACCTCGGTTCAAAGAGTTGGTAGCTCAGAGAACCTTCGAAAAACCGC CCTGCAAGGCGGTTTTTTCGTTTTCAGAGCAAGAGATTACGCGCAGACCAAAACGATCTCAA GAAGATCATCTTATTAATCAGATAAAATATTTCTAGATTTCAGTGCAATTTATCTCTTCAAATGT AGCACCTGAAGTCAGCCCCATACGATATAAGTTGTAATTCTCATGTTTGACAGCTTATCATCG ATAAGCTTCCGATGGCGCGCCGAGAGGCTTTACACTTTATGCTTCCGGCT
Example STAR (Variant 1) DNA Plasmid: (Promoter 1-STAR 1 - t500-ColE1 origin - AmpR)	GAATTCTAAAGATCTTTGACAGCTAGCTCAGTCCTAGGTATAATACTAGTTGAACTGTATACAT TCCCCGCAGGATGAGATGAGAACGATAGAGATGCAAAGGTAAGATGGGGATCTCAAAGCCC GCCGAAAGGCGGGCTTTTTTTTGGATCCTTACTCGAGTCTAGACTGCAGGCTTCCTCGCTCA CTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGT AATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGC AAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCACAGGCTCCGCCCCCC TGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAA GATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTT ACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTG TAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCC GTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACA CGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGC GGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGG TATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCA AACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAA AAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAA CTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAAT TAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAAT GCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGAC TCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATG ATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAA GGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGC CGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTAC AGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGAT CAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCG ATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAAT TCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCA TTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATAC CGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAAC TCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGAT CTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCC GCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATAT TATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAA ATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACC ATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCAGAATTTCAGATAAAAAA AATCCTTAGCTTTCGCTAAGGATGATTTCTG
Example AHL inducible STAR (Variant 5) DNA Plasmid: (ptet-	GAATTCTAAAGATCTTCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACT GAGCACTACTAGAGAAAGAGGAGAAATACTAGATGAAAAACATAAATGCCGACGACACATAC AGAATAATTAATAAAATTAAAGCTTGTAGAAGCAATAATGATATTAATCAATGCTTATCTGATAT GACTAAAATGGTACATTGTGAATATTATTTACTCGCGATCATTTATCCTCATTCTATGGTTAAAT CTGATATTTCAATCCTAGATAATTACCCTAAAAAATGGAGGCAATATTATGATGACGCTAATTT AATAAAATATGATCCTATAGTAGATTATTCTAACTCCAATCATTCACCAATTAATTGGAATATAT TTGAAAACAATGCTGTAAATAAAAAATCTCCAAATGTAATTAAAGAAGCGAAAACATCAGGTCT TATCACTGGGTTTAGTTTCCCTATTCATACGGCTAACAATGGCTTCGGAATGCTTAGTTTTGC ACATTCAGAAAAAGACAACTATATAGATAGTTTATTTTTACATGCGTGTATGAACATACCATTA ATTGTTCCTTCTCTAGTTGATAATTATCGAAAAATAAATATAGCAAATAATAAATCAAACAACGA TTTAACCAAAAGAGAAAAAGAATGTTTAGCGTGGGCATGCGAAGGAAAAAGCTCTTGGGATA

B0034-LuxR-B0015-plux-STAR5 -t500-ColE1 origin -AmpR)	TTTCAAAAATATTAGGTTGCAGTGAGCGTACTGTCACTTTCCATTTAACCAATGCGCAAATGAA ACTCAATACAACAAACCGCTGCCAAAGTATTTCTAAAGCAATTTTAACAGGAGCAATTGATTG CCCATACTTTAAAAATTAATAACACTGATAGTGCTAGTGTAGATCACTACTAGAGCCAGGCAT CAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTG AACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTA GAGACCTGTAGGATCGTACAGGTTTACGCAAGAAAATGGTTTGTTATAGTCGAATAAATGAAC TGTATACATTCCCCGCAGGATAGGAATTGAAGATGAAACGATGAGACTTGGGACGAGGATCT CAAAGCCCGCCGAAAGGCGGGCTTTTTTTTGGATCCTTACTCGAGTCTAGACTGCAGGCTTC CTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCA AAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAA AGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCACAGGCTC CGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAG GACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACC CTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAG CTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACG AACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCG GTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGT ATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACA GTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGA TCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCG CAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAA CGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCT TTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTT ACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTG CCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCT GCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGC CGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATT GTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATT GCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCA ACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTC CTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTG CATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACC AAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGA TAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGC GAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCC AACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAA AATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTT CAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTT AGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAA GAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCAGAATTTCAGAT AAAAAAAATCCTTAGCTTTCGCTAAGGATGATTTCTG
Example Target (Variant 5) sgRNA DNA Plasmid: (Promoter 3 – Target 5 – Csy4 hairpin – sgRNA – TrrnB – SpecR – CDF origin)	GAATTCTAAAGATCTTTGACAATTAATCATCCGGCTCGTAATTTATGTGGTCGTCCCAAGTCTC ATCGTTTCATCTTCAATTCCTATCCTGCGGGGAATGTATACAGTTCATGTATATATTCCCCGCT TTTTTTTTGTTCACTGCCGTATAGGCAGCTAAGAAATGGAACCGTACTGGAACTGCGTTTAAG AGCTATGCTGGAAACAGCATAGCAAGTTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGT GGCACCGAGTCGGTGCTTTTTTTGAAGCTTGGGCCCGAACAAAAACTCATCTCAGAAGAGGA TCTGAATAGCGCCGTCGACCATCATCATCATCATCATTGAGTTTAAACGGTCTCCAGCTTGGC TGTTTTGGCGGATGAGAGAAGATTTTCAGCCTGATACAGATTAAATCAGAACGCAGAAGCGG TCTGATAAAACAGAATTTGCCTGGCGGCAGTAGCGCGGTGGTCCCACCTGACCCCATGCCG AACTCAGAAGTGAAACGCCGTAGCGCCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTAG GGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTAT CTGTTGTTTGTCGGTGAACTGGATCCTTACTCGAGTCTAGACTGCAGCTGAAACCTCAGGCA TTTGAGAAGCACACGGTCACACTGCTTCCGGTAGTCAATAAACCGGTAAACCAGCAATAGAC ATAAGCGGCTATTTAACGACCCTGCCCTGAACCGACGACCGGGTCATCGTGGCCGGATCTT GCGGCCCCTCGGCTTGAACGAATTGTTAGACATCGCCTTTCACGTAGTGGACAAATTCTTCC AACTGATCTGCGCGCGAGGCCAAGCGATCTTCTTCTTGTCCAAGATAAGCCTGTCTAGCTTC AAGTATGACGGGCTGATACTGGGCCGGCAGGCGCTCCATTGCCCAGTCGGCAGCGACATCC TTCGGCGCTTATTTGCCGACTACCTTGGTGATCGATTTTGCCGGTTACTGCGCTGTACCAAAT GCGGGACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTCCAT AGCGTTAAGGTTTCATTTAGCGCCTCAAATAGATCCTGTTCAGGAACCGGATCAAAGAGTTCC TCCGCCGCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGTCAGCAAGATAGCCAG ATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATTGCGCTGCCATTCTC CAAATTGCAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGCACAACAATG GTGACTTCTACAGCGCGGAGAATCTCGCTCTCTCCAGGGGAAGCCGAAGTTTCCAAAAGGTC GTTGATCAAAGCTCGCCGCGTTGTTTCATCAAGCCTTACGGTCACCGTAACCAGCAAATCAAT ATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCCGTACAAATGTACGGCCAGCAAC GTCGGTTCGAGATGGCGCTCGATGACGCCAACTACCTCTGATAGTTGAGTCGATACTTCGGC GATCACCGCTTCCCTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGT CTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGCTAGCTCACTCGGT CGCTACGCTCCGGGCGTGAGACTGCGGCGGGCGCTGCGGACACATACAAAGTTACCCACA

	GATTCCGTGGATAAGCAGGGGACTAACATGTGAGGCAAAACAGCAGGGCCGCGCCGGTGG CGTTTTTCCATAGGCTCCGCCCCTCCTGCCAGAGTTCACATAAACAGACGCTTTTCCGGTGCA TCTGTGGGAGCCGTGAGGCTCAACCATGAATCTGACAGTACGGGCGAAACCCGACAGGACT TAAAGATCCCCACCGTTTCCGGCGGGTCGCTCCCTCTTGCGCTCTCCTGTTCCGACCCTGCC GTTTACCGGATACCTGTTCCGCCTTTCTCCCTTACGGGAAGTGTGGCGCTTTCTCATAGCTCA CACACTGGTATCTCGGCTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTAAGCAAGAACTC CCCGTTCAGCCCGACTGCTGCGCCTTATCCGGTAACTGTTCACTTGAGTCCAACCCGGAAAA GCACGGTAAAACGCCACTGGCAGCAGCCATTGGTAACTGGGAGTTCGCAGAGGATTTGTTTA GCTAAACACGCGGTTGCTCTTGAAGTGTGCGCCAAAGTCCGGCTACACTGGAAGGACAGATT TGGTTGCTGTGCTCTGCGAAAGCCAGTTACCACGGTTAAGCAGTTCCCCAACTGACTTAACC TTCGATCAAACCACCTCCCCAGGTGGTTTTTTCGTTTACAGGGCAAAAGATTACGCGCAGAAA AAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACTGAACCGCTCTAGATTTCAGTGCAATTT ATCTCTTCAAATGTAGCACCTGAAGTCAGCCCCATACGATATAAGTTGTAATTCTCATGTTAGT CATGCCCCGCGCCCACCGGAAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATCGGTCG AGATCCCGGTGCCTAATGAGTGAGCTAACTTACATTAATTGCGTTGCGC
Example sgRNA and Target (Variant 5) mRFP DNA Plasmid: (Promoter 3 – sgRNA – TrrnB – SpecR – CDF origin – Promoter 1 – Target 5 – RBS_RFP – mRFP – TrrnB)	GAATTCTAAAGATCTTTGACAATTAATCATCCGGCTCGTAATTTATGTGGTGGAACCGTACTG GAACTGCGTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTAAATAAGGCTAGTCCGTTAT CAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTGAAGCTTGGGCCCGAACAAAAACTC ATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATTGAGTTTAAACG GTCTCCAGCTTGGCTGTTTTGGCGGATGAGAGAAGATTTTCAGCCTGATACAGATTAAATCAG AACGCAGAAGCGGTCTGATAAAACAGAATTTGCCTGGCGGCAGTAGCGCGGTGGTCCCACC TGACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGCGCCGATGGTAGTGTGGGGTCTCCC CATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGG GCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACTGGATCCTTACTCGAGTCTAGACTGCAGCT GAAACCTCAGGCATTTGAGAAGCACACGGTCACACTGCTTCCGGTAGTCAATAAACCGGTAA ACCAGCAATAGACATAAGCGGCTATTTAACGACCCTGCCCTGAACCGACGACCGGGTCATCG TGGCCGGATCTTGCGGCCCCTCGGCTTGAACGAATTGTTAGACATTATTTGCCGACTACCTT GGTGATCTCGCCTTTCACGTAGTGGACAAATTCTTCCAACTGATCTGCGCGCGAGGCCAAGC GATCTTCTTCTTGTCCAAGATAAGCCTGTCTAGCTTCAAGTATGACGGGCTGATACTGGGCC GGCAGGCGCTCCATTGCCCAGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTACTG CGCTGTACCAAATGCGGGACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGG CGGCGAGTTCCATAGCGTTAAGGTTTCATTTAGCGCCTCAAATAGATCCTGTTCAGGAACCG GATCAAAGAGTTCCTCCGCCGCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGTC AGCAAGATAGCCAGATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATT GCGCTGCCATTCTCCAAATTGCAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGT CGTGCACAACAATGGTGACTTCTACAGCGCGGAGAATCTCGCTCTCTCCAGGGGAAGCCGA AGTTTCCAAAAGGTCGTTGATCAAAGCTCGCCGCGTTGTTTCATCAAGCCTTACGGTCACCG TAACCAGCAAATCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCCGTACAAA TGTACGGCCAGCAACGTCGGTTCGAGATGGCGCTCGATGACGCCAACTACCTCTGATAGTT GAGTCGATACTTCGGCGATCACCGCTTCCCTCATACTCTTCCTTTTTCAATATTATTGAAGCAT TTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATA GCTAGCTCACTCGGTCGCTACGCTCCGGGCGTGAGACTGCGGCGGGCGCTGCGGACACAT ACAAAGTTACCCACAGATTCCGTGGATAAGCAGGGGACTAACATGTGAGGCAAAACAGCAGG GCCGCGCCGGTGGCGTTTTTCCATAGGCTCCGCCCTCCTGCCAGAGTTCACATAAACAGAC GCTTTTCCGGTGCATCTGTGGGAGCCGTGAGGCTCAACCATGAATCTGACAGTACGGGCGA AACCCGACAGGACTTAAAGATCCCCACCGTTTCCGGCGGGTCGCTCCCTCTTGCGCTCTCCT GTTCCGACCCTGCCGTTTACCGGATACCTGTTCCGCCTTTCTCCCTTACGGGAAGTGTGGCG CTTTCTCATAGCTCACACACTGGTATCTCGGCTCGGTGTAGGTCGTTCGCTCCAAGCTGGGC TGTAAGCAAGAACTCCCCGTTCAGCCCGACTGCTGCGCCTTATCCGGTAACTGTTCACTTGA GTCCAACCCGGAAAAGCACGGTAAAACGCCACTGGCAGCAGCCATTGGTAACTGGGAGTTC GCAGAGGATTTGTTTAGCTAAACACGCGGTTGCTCTTGAAGTGTGCGCCAAAGTCCGGCTAC ACTGGAAGGACAGATTTGGTTGCTGTGCTCTGCGAAAGCCAGTTACCACGGTTAAGCAGTTC CCCAACTGACTTAACCTTCGATCAAACCACCTCCCCAGGTGGTTTTTTCGTTTACAGGGCAAA AGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACTGAACCGCTCT AGATTTCAGTGCAATTTATCTCTTCAAATGTAGCACCTGAAGTCAGCCCCATACGATATAAGTT GTAATTCTCATGTTAGTCATGCCCCGCGCCCACCGGAAGGAGCTGACTGGGTTGAAGGCTCT CAAGGGCATCGGTCGAGATCCCGGTGCCTAATGAGTGAGCTAACTTACATTAATTGCGTTGC GCAAATGTAGCACCTGAAGTCAGCCCCATACGATATAAGTTGTAATTCTCATGTTTGACAGCT TATCATCGATAAGCTTCCGATGGCGCGCCGAGAGGCTTTACACTTTATGCTTCCGGCTGAAT TCTAAAGATCTTTGACAGCTAGCTCAGTCCTAGGTATAATACTAGTTCGTCCCAAGTCTCATC GTTTCATCTTCAATTCCTATCCTGCGGGGAATGTATACAGTTCATGTATATATTCCCCGCTTTT TTTTTGGATCTGAATTCATTAAAGAGGAGAAAGGTACCATGGCGAGTAGCGAAGACGTTATCA AAGAGTTCATGCGTTTCAAAGTTCGTATGGAAGGTTCCGTTAACGGTCACGAGTTCGAAATC GAAGGTGAAGGTGAAGGTCGTCCGTACGAAGGTACCCAGACCGCTAAACTGAAAGTTACCA AAGGTGGTCCGCTGCCGTTCGCTTGGGACATCCTGTCCCCGCAGTTCCAGTACGGTTCCAA AGCTTACGTTAAACACCCGGCTGACATCCCGGACTACCTGAAACTGTCCTTCCCGGAAGGTT TCAAATGGGAACGTGTTATGAACTTCGAAGACGGTGGTGTTGTTACCGTTACCCAGGACTCC TCCCTGCAAGACGGTGAGTTCATCTACAAAGTTAAACTGCGTGGTACCAACTTCCCGTCCGA CGGTCCGGTTATGCAGAAAAAAACCATGGGTTGGGAAGCTTCCACCGAACGTATGTACCCG GAAGACGGTGCTCTGAAAGGTGAAATCAAAATGCGTCTGAAACTGAAAGACGGTGGTCACTA

	CGACGCTGAAGTTAAAACCACCTACATGGCTAAAAAACCGGTTCAGCTGCCGGGTGCTTACA AAACCGACATCAAACTGGACATCACCTCCCACAACGAAGACTACACCATCGTTGAACAGTAC GAACGTGCTGAAGGTCGTCACTCCACCGGTGCTTAATAAGGATCTGAAGCTTGGGCCCGAA CAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATTGA GTTTAAACGGTCTCCAGCTTGGCTGTTTTGGCGGATGAGAGAAGATTTTCAGCCTGATACAG ATTAAATCAGAACGCAGAAGCGGTCTGATAAAACAGAATTTGCCTGGCGGCAGTAGCGCGGT GGTCCCACCTGACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGCGCCGATGGTAGTGTG GGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCG AAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACTGGATCCTTACTCGAGTCTAG ACTGCAGTTG

Supplementary Table 3. Sequence of Promoter and RBS variants. Promoter 1 (BBa_J23119_SpeI) is a variant of BBa_J23119 obtained from the iGEM Registry of Standard Biological Parts (parts.igem.org). Promoters 2-5 were derived from a previously published promoter library[5]. RBS variants were generated by inverse PCR using degenerate oligonucleotides and five variants chosen that demonstrated distinct expression strengths of sfGFP from target variant 5 in the presence of cognate STAR. The stability hairpin used for the no target RNA control in **Supplementary Fig. 12**. was derived from a previously published library[6].

Name	Sequence (5' to 3')
Promoter 1	TTGACAGCTAGCTCAGTCCTAGGTATAATACTAGT
Promoter 2	AAAAAGAGTATTGACTTCGCATCTTTTTGTACCTATAATGTGTGG
Promoter 3	TTGACAATTAATCATCCGGCTCGTAATTTATGTGG
Promoter 4	AAAAAATTTATTTCGCATCTTTTTGTACCTATAATGTGTGG
Promoter 5	TTGACAATTAATCATCCGGCTCGTAGGGTTTGTGG
RBS 1	AGGAGGAA
RBS 2	GTAACGGA
RBS 3	GTATTGGA
RBS 4	TATTGGGA
RBS 5	TAGAGGTG
Stability hairpin	ACGTCGACTCTCGAGTGAGATTGTTGACGGTACCGTATTTT

Supplementary Table 4. Sequences of NUPACK-designed targets and target length variants used in this study. Plasmid sequences can be constructed by replacing the pink region in the example target plasmid in **Supplementary Table 2** with the sequences below. Red highlighting indicates nucleotide insertions or mutations that resulted from cloning.

Plasmid #	Target sequence (5' to 3')	Variant #
pJBL4900	AGACAACGAAGATAAAGACAGGACAAGGACAGCACGCGATGCGGGGAA TGTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	77
pJBL4902	TCGTTCGTCCATCTCAATTTCAATACTCCATGCTTCTACTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	32
pJBL4904	CAAGAACAGACGACCAGGACGAGCAGTAGATTAGCATTATGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	81
pJBL4906	CCTTATCCTTATCTTACCTCATTTCTATCACTCTATTCTTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	4
pJBL4908	CCTCGCCCATCTCATTATCTTCACATCTCATCTCATCCTTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	29
pJBL4910	GAAGATAACGCAAGAACGAACGAAACTTTAGGGCCATGGTGCGGGGAAT	76

	GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	
pJBL4912	GGAGCAATGAGGATAGGAACGATAGTAGACGGACGGGTATGCGGGGAA TGTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	98
pJBL4916	CTCTCATATTCTCATTTCGCTCTCACCTATCTATCTCTGTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	20
pJBL4918	CTCATCTCATTTCGCTCTCACATTTCTACACCTTTATCTTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	10
pJBL4920	CAAAGCAGGCGAATAAGGCATCAAATCAATCATCACTATTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	93
pJBL4922	GCCTCCAATTCCAATCTTCATCCCATTATCTTATCTCGTTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	22
pJBL4924	GATATGGCATGTTCCTAAGGCCTCCGCGTTTGATTCTTGTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	82
pJBL4926	GGGACTAACTACTATGAACTTGACGAACTATGATGCTGCTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	56
pJBL4928	CCCTCACCTCCATCTCTATCTATCGCTTCCATCTTACTCTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	7
pJBL4930	CCAAGACATAAAGATAAAGACAAATAGCAACGACCGACCTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	37
pJBL4932	CCTTCAATTTCCATCCTATCTTATTCTTATCTTGCCTGTTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	12
pJBL4934	GAGACAAGAATAGAACACGAGCAAAGATAGACGACGACCTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	73
pJBL4936	GGGCCTTCGATTTACCAGCTTTCAGATTTCAATTACGTGTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	74
pJBL4938	AACTCCTATCCTGCTGCCTATCTGTCTCTATCTCTATCCTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	15
pJBL4940	CCTTATTCCATTCACTTATTCCTACTTACTTGCCTCTATTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	51
pJBL4942	CTCCCGCTCTATTCCTACTTATCCTCATCTCCATCTACCTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	43
pJBL4944	GAAACCTGAAATGACGAAACGCTGAAATTGCGATATACCTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	63
pJBL4946	GAGTGAAAGTAGAATAAGATAAAGCGAACCGGGACCATATGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	19
pJBL4948	GGGTGAGAGTGGAGTGGAGTGGGAGTAGGGTAGGGCATGTGCGGGGA ATGTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	94
pJBL4950	CATATTCCATTCCATTTTGTTCCAGTTTCAGTCATTTCGTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTTT	101
pJBL4952	CCAGAAACATAAAGATAACACGACGCACAGATAGGCACTTTGCGGGGAA TGTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	50
pJBL4954	CATCATAGTCCAAGTTCAAGTTTCCATCATCAGTTCCGTTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	9
pJBL4956	TCAATCAAAGTACAGCGTCAAGTCAAATCAGCATATTCGTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	90
pJBL4958	CCAATAGTCCAAGTTCGCCAAGTTCATATAGTCGTTTCATGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	27
pJBL4960	GTCCTAATTCTCATCGTCTATCTTCATCTATCTCTATCTTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	11
pJBL4962	GACCTAGACTGATGAAACGACGATAATGATAACACTTGCTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	95
pJBL4964	GGGACCGAGAGCAATAGAGATTTAGATACAGACAACTTATGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	96
pJBL4966	GGGAGTGGGAGTTGGAGATGTGCGGAGACGGATGGTATTTGCGGGGAA TGTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	99
pJBL4968	CTCTCATTCTATTCATCTCATCTATTCTTATCATCCATCTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	34
pJBL4970	TCGTCCCAAGTCTCATCGTTTCATCTTCAATTCCTATCCTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	5
pJBL4972	CCACTCACTTCTACTCATCTCATCTTCACTTCATCCACTTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	25
pJBL4974	CCCTTACTCTCATTGCTCTCACTCGTTCTATCTATCGTTTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	18
pJBL4976	CCCTATTTCGATCTACTTAAAGCCTCATCATTTCTGCGTTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	35
pJBL4978	CCTCTATCCTATCTATCTGCCTGTCCTGTCCTGTGTCTTTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	3
pJBL4980	CCAGTCCTCAAGTTTCGTTCAATCCATCCTATCCTATTCTGCGGGGAATG	14

	TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	
pJBL4982	CAACAGAAATCAACTTAGGACTATACGGCGATACGAGCTTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	88
pJBL4984	GAAAGCTGATAGAAACGAGATGAACATTTGAGGACGACTTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	55
pJBL4986	GCCCTATCCCATCATCTCACTATCTCATCTTCATCTCACTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	57
pJBL4988	GAAACCTAAGAATATGATAACACTAACTAACGACGGCACTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	69
pJBL4990	CCTCACCTCATTATCTTACCATTTCATCTTTACTTCACTTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	44
pJBL4992	TCCCGCTTCTACCTATCTCTACTCATTTCTATTCATCTCTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	87
pJBL4994	GTCCTCGTTCGTTTCCAGTTTATCCTATCATGTTTCCGTTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	92
pJBL4996	CTCACCCTATCTCGCTTCATTTACTTATCCTACCTCATCTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	60
pJBL4998	ACACAGAGCAAACGAGACAAGACAGGAACACGACGATTATGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	83
pJBL5802	CTATGACTAACTAAATGAACGAATGAACTACTGACTGACTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	70
pJBL5804	CTATCTGCCTTCAATTGTTCCGTCTCTATCATCTTTATCTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	65
pJBL5806	GTCCAGTCCATAGTATTCTCAAGTTCCCATTTCGTTCGTTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	66
pJBL5808	CCATCCTCAATCTCTACCTACTCTCACTTACTCTTATCCTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	8
pJBL5812	GAACACTTACTTACGACGGCTACTTATCTCTATTTCTATTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	49
pJBL5814	GTTCAGTATCCTATCTTCAATCAAATGTCTTTATCTCGGTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	75
pJBL5816	CCAGTCATCAAGTCAGTCCAGTCAAAGTTTCCGTCGTTCAGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	6
pJBL5818	CAATATCCCGCTCCTATCTCCAATCCTATCTACTCACTCTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	31
pJBL5820	TCATAATCTCATCTATCCTTGCCCAATGTTCTTAATCCTTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	48
pJBL5822	TGCCCTGTCCTATCTTTATGTCCTGTCCTGTCTTGTCCTTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	23
pJBL5824	CATACGAAGTCCATAGAGTTTATCAAGCCGGTTGCCATTTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	86
pJBL5826	GAATAACAATGACAACGACGAAATAGACCACGATACCTTTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	46
pJBL5828	TCATCCATCCTTACCTTACCTATCTATTCTCATCTCTACTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	52
pJBL5830	CTCTCAACTCACCTCTATCTATCTATCTCTATGCTCTCGTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	53
pJBL5832	CCTCATCATCGTCCTCATCTATTTCCATCTACTTATCTCTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	21
pJBL5834	TCTTATCACCCTTATTCCATTAACTTCTGCCTTGTCTCGTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	17
pJBL5836	GACGAGAATTACAGCAGAAACAGAAACGACAAGCACTTATGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	68
pJBL5838	GAAACATAGATAAGCAAAGATAAGCAAGAATTCATAACCTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	84
pJBL5840	CTCGCCACCTCACTTATCCTCATCCTATCTCACCTCATCTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	39
pJBL5842	CCTCGCTCTCATTTCATCTCATCATCTCATATTCCCATCTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	24
pJBL5844	CCCATCTCATCTCATTCTCATCTCATTCATCTATCTCGTTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	13
pJBL5846	GACAAGAATAAGAACAAGACAGAATACGATACCGAACTATGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	36
pJBL5848	GACAAATAGAAACAGAAACGACGGACATAACATACATACTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	72
pJBL5850	CTCATTTCATTTCATCATCTATCTCTACCCATCTATTCTTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	26
pJBL5852	GCCCTCATCTCATCAATCCTCAATCCCTATCTTCGTCTCTGCGGGGAATG	80

	TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	
pJBL5854	CCCACTCTCATCTTACCTCTACCTATTCTATTCTTGTCCTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	54
pJBL5856	GTCCCTATCCATTTCATATCTTACTTATCTCTGCATTCTTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	79
pJBL5860	GAATGATAAGAACGCAAATGAATACGATAGACTGGACTTTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	71
pJBL5864	CATAAGCCAGTCATAAGTAAAGTCAATAGTCGTCCATCCTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	42
pJBL5866	CAATCTTCAATCTCCATCTCATCCATCTTTATCTCGTCTTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	30
pJBL5868	CCATCTTACCTTTGCATCTCTATCGTTCTCATCTCATCCTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	1
pJBL5870	CAAATCTATCCGCCTCTAATCTTCATCTCTACCTGTCTCTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	91
pJBL5874	CCACCTTAACTCTCACGACTGCTGCCTTGCCTATTGCTTTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	38
pJBL5876	TCACAGTCACGAATAAGCAGCCTCCAATATCATCATCTTTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	45
pJBL5878	CTCACCTCGCCTCTATCCTTACCATCTATCTATCTCTGTTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	67
pJBL5880	CCTCATCCTTATCTTGCTCCTATTGTCCATTTCTATCTCTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	16
pJBL5882	CCTCACCTTCATCTTCATCTTCATCTTTACCATTCATCGTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	47
pJBL5884	GGATAGAGCGGAGACAAGGTAGGGTAGGGATGGTAGTATTGCGGGGAA TGTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	97
pJBL5886	GGAATATGAATGAATGGGAACTTGGGAACTTGACTTTGATGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	100
pJBL5888	CAGTAGTCAAAGTCCAGTAAGTCCTCAGTTCCCGTTCGTTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	89
pJBL5890	CAATAACAAGCAAGCAAAGACAATAGAATAACGGGACCATGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	33
pJBL5892	CATCTCCACTCACCTCTACTTATCTATTCTTACTGTCCTTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	78
pJBL5894	GTCTCCAATTCTTAATCCCGCTACCTATCTCGTCCACTTTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	58
pJBL5896	CTCGCCTTACAATACCAGTTTCAATCTTCAATATGTCCTTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	64
pJBL5898	CGCTCTTATTCATCTCCATTCGTCTCATCTATCTCCGTCTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	41
pJBL5900	CCTCCATCTCCATATTCTTATCTCATTATCATCTCACTTTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	2
pJBL4144	CTTTCGCGTTTCATTTCATTATCTTATCGTCTTTGTCTTTGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	62
pJBL4146	CCTATTTGCTTTATCATCTCATTTCATTATCTTGTTCTTAGCGGGGAATGT ATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	59
pJBL4148	CTCTATCTCGCTCATCTATCACTCTCATCTCATCCTCACTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	40
pJBL4150	CTCCTACCCATTTCTACCTCACTTACTCACTACTTCTACTGCGGGGAATG TATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	61
pJBL4152	CGCCTACTCCACTTGTTCGAAACCGGCTGCTTTACACGGTGCGGGGAAT GTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	85
pJBL2801	AGTTTTTACAGTGAATTGTTTTAATTAGTTGTATAAATGTTGGAGCAGCGG GGAATGTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT	WT and 28 and length variant used in SI 1
pJBL2802	GTCTAGGAAAAGTTTTTACAGTGAATTGTTTTAATTAGTTGTATAAATGTT GGAGCAGCGGGGAATGTATACAGTTCATGTATATATTCCCCGCTTTTTTT TT	Length variant used in SI 1
pJBL6066	CTCTATCTCTGTGTCTCCAATTTGTCCTGTCCTGTCTCTGATTTGCTTGCC GGAAGCAAAATAACCGGCAAGCAAATAGTTGTTACT	RibA 1
pJBL6065	CTCCCATCCCATCTCATTCATCCACTCTACTCTCATCCCTATTTGCTTGCC GGAAGCAAAATAACCGGCAAGCAAATAGTTGTTACT	RibA 2
pJBL6067	GCATACTACGGGACAACGGGACGGACTACAAGAACTTAAGATTTGCTTG CCGGAAGCAAAATAACCGGCAAGCAAATAGTTGTTACT	RibA 3
pJBL6068	GTTAGGGTCAAGGTGTAGGGTAGGTAGGTAGCGTAAGCGTATTTGCTTG	RibA 4

	CCGGAAGCAAAATAACCGGCAAGCAAATAGTTGTTACT	
pJBL6069	GAGACAAGTAAACGATAAGAACAGAACATAGGGCGACGGCATTTGCTTG CCGGAAGCAAAATAACCGGCAAGCAAATAGTTGTTACT	RibA 5

Supplementary Table 5. Sequences of NUPACK-designed STARs and STAR length variants used in this study. Plasmid sequences can be constructed by replacing the dark blue region in the example STAR plasmid in **Supplementary Table 2** with the sequences below.

Plasmid #	Target sequence (5' to 3')	Variant #
pJBL4901	TGAACTGTATACATTCCCCGCATCGCGTGCTGTCCTTGTCCTGTCTTTATCTTCGTTGTCT	77
pJBL4903	TGAACTGTATACATTCCCCGCAGTAGAAGCATGGAGTATTGAAATTGAGATGGACGAACGA	32
pJBL4905	TGAACTGTATACATTCCCCGCATAATGCTAATCTACTGCTCGTCCTGGTCGTCTGTTCTTG	81
pJBL4907	TGAACTGTATACATTCCCCGCAAGAATAGAGTGATAGAAATGAGGTAAGATAAGGATAAGG	4
pJBL4909	TGAACTGTATACATTCCCCGCAAGGATGAGATGAGATGTGAAGATAATGAGATGGGCGAGG	29
pJBL4911	TGAACTGTATACATTCCCCGCACCATGGCCCTAAAGTTTCGTTCGTTCTTGCGTTATCTTC	76
pJBL4913	TGAACTGTATACATTCCCCGCATACCCGTCCGTCTACTATCGTTCCTATCCTCATTGCTCC	98
pJBL4917	TGAACTGTATACATTCCCCGCACAGAGATAGATAGGTGAGAGCGAAATGAGAATATGAGAG	20
pJBL4919	TGAACTGTATACATTCCCCGCAAGATAAAGGTGTAGAAATGTGAGAGCGAAATGAGATGAG	10
pJBL4921	TGAACTGTATACATTCCCCGCAATAGTGATGATTGATTTGATGCCTTATTCGCCTGCTTTG	93
pJBL4923	TGAACTGTATACATTCCCCGCAACGAGATAAGATAATGGGATGAAGATTGGAATTGGAGGC	22
pJBL4925	TGAACTGTATACATTCCCCGCACAAGAATCAAACGCGGAGGCCTTAGGAACATGCCATATC	82
pJBL4927	TGAACTGTATACATTCCCCGCAGCAGCATCATAGTTCGTCAAGTTCATAGTAGTTAGTCCC	56
pJBL4929	TGAACTGTATACATTCCCCGCAGAGTAAGATGGAAGCGATAGATAGAGATGGAGGTGAGGG	7
pJBL4931	TGAACTGTATACATTCCCCGCAGGTCGGTCGTTGCTATTTGTCTTTATCTTTATGTCTTGG	37
pJBL4933	TGAACTGTATACATTCCCCGCAACAGGCAAGATAAGAATAAGATAGGATGGAAATTGAAGG	12
pJBL4935	TGAACTGTATACATTCCCCGCAGGTCGTCGTCTATCTTTGCTCGTGTTCTATTCTTGTCTC	73
pJBL4937	TGAACTGTATACATTCCCCGCACACGTAATTGAAATCTGAAAGCTGGTAAATCGAAGGCCC	74
pJBL4939	TGAACTGTATACATTCCCCGCAGGATAGAGATAGAGACAGATAGGCAGCAGGATAGGAGTT	15
pJBL4941	TGAACTGTATACATTCCCCGCAATAGAGGCAAGTAAGTAGGAATAAGTGAATGGAATAAGG	51
pJBL4943	TGAACTGTATACATTCCCCGCAGGTAGATGGAGATGAGGATAAGTAGGAATAGAGCGGGAG	43
pJBL4945	TGAACTGTATACATTCCCCGCAGGTATATCGCAATTTCAGCGTTTCGTCATTTCAGGTTTC	63
pJBL4947	TGAACTGTATACATTCCCCGCATATGGTCCCGGTTCGCTTTATCTTATTCTACTTTCACTC	19
pJBL4949	TGAACTGTATACATTCCCCGCACATGCCCTACCCTACTCCCACTCCACTCCACTCTCACCC	94
pJBL4951	TGAACTGTATACATTCCCCGCACGAAATGACTGAAACTGGAACTAAATGGAATGGAATATG	101
pJBL4953	TGAACTGTATACATTCCCCGCAAAGTGCCTATCTGTCGTCGTGTTATCTTTATGTTTCTGG	50
pJBL4955	TGAACTGTATACATTCCCCGCAACGGAACTGATGATGGAAACTTGAACTTGGACTATGATG	9
pJBL4957	TGAACTGTATACATTCCCCGCACGAATATGCTGATTTGACTTGACGCTGTACTTTGATTGA	90
pJBL4959	TGAACTGTATACATTCCCCGCATGAAACGACTATATGAACTTGGCGAACT	27

	TGGACTATTGG	
pJBL4961	TGAACTGTATACATTCCCCGCAAGATAGAGATAGATGAAGATAGACGATG AGAATTAGGAC	11
pJBL4963	TGAACTGTATACATTCCCCGCAGCAAGTGTTATCATTATCGTCGTTTCATC AGTCTAGGTC	95
pJBL4965	TGAACTGTATACATTCCCCGCATAAGTTGTCTGTATCTAAATCTCTATTGC TCTCGGTCCC	96
pJBL4967	TGAACTGTATACATTCCCCGCAAATACCATCCGTCTCCGCACATCTCCAA CTCCCACTCCC	99
pJBL4969	TGAACTGTATACATTCCCCGCAGATGGATGATAAGAATAGATGAGATGAA TAGAATGAGAG	34
pJBL4971	TGAACTGTATACATTCCCCGCAGGATAGGAATTGAAGATGAAACGATGAG ACTTGGGACGA	5
pJBL4973	TGAACTGTATACATTCCCCGCAAGTGGATGAAGTGAAGATGAGATGAGTA GAAGTGAGTGG	25
pJBL4975	TGAACTGTATACATTCCCCGCAAACGATAGATAGAACGAGTGAGAGCAAT GAGAGTAAGGG	18
pJBL4977	TGAACTGTATACATTCCCCGCAACGCAGAAATGATGAGGCTTTAAGTAGA TCGAAATAGGG	35
pJBL4979	TGAACTGTATACATTCCCCGCAAAGACACAGGACAGGACAGGCAGATAG ATAGGATAGAGG	3
pJBL4981	TGAACTGTATACATTCCCCGCAGAATAGGATAGGATGGATTGAACGAAAC TTGAGGACTGG	14
pJBL4983	TGAACTGTATACATTCCCCGCAAGCTCGTATCGCCGTATAGTCCTAAGTT GATTTCTGTTG	88
pJBL4985	TGAACTGTATACATTCCCCGCAAGTCGTCCTCAAATGTTCATCTCGTTTCT ATCAGCTTTC	55
pJBL4987	TGAACTGTATACATTCCCCGCAGTGAGATGAAGATGAGATAGTGAGATGA TGGGATAGGGC	57
pJBL4989	TGAACTGTATACATTCCCCGCAGTGCCGTCGTTAGTTAGTGTTATCATAT TCTTAGGTTTC	69
pJBL4991	TGAACTGTATACATTCCCCGCAAGTGAAGTAAAGATGAAATGGTAAGATA ATGAGGTGAGG	44
pJBL4993	TGAACTGTATACATTCCCCGCAGAGATGAATAGAAATGAGTAGAGATAGG TAGAAGCGGGA	87
pJBL4995	TGAACTGTATACATTCCCCGCAACGGAAACATGATAGGATAAACTGGAAA CGAACGAGGAC	92
pJBL4997	TGAACTGTATACATTCCCCGCAGATGAGGTAGGATAAGTAAATGAAGCGA GATAGGGTGAG	60
pJBL4999	TGAACTGTATACATTCCCCGCATAATCGTCGTGTTCCTGTCTTGTCTCGT TTGCTCTGTGT	83
pJBL5803	TGAACTGTATACATTCCCCGCAGTCAGTCAGTAGTTCATTCGTTCATTTA GTTAGTCATAG	70
pJBL5805	TGAACTGTATACATTCCCCGCAGATAAAGATGATAGAGACGGAACAATTG AAGGCAGATAG	65
pJBL5807	TGAACTGTATACATTCCCCGCAACGAACGAAATGGGAACTTGAGAATACT ATGGACTGGAC	66
pJBL5809	TGAACTGTATACATTCCCCGCAGGATAAGAGTAAGTGAGAGTAGGTAGA GATTGAGGATGG	8
pJBL5813	TGAACTGTATACATTCCCCGCAATAGAAATAGAGATAAGTAGCCGTCGTA AGTAAGTGTTC	49
pJBL5815	TGAACTGTATACATTCCCCGCACCGAGATAAAGACATTTGATTGAAGATA GGATACTGAAC	75
pJBL5817	TGAACTGTATACATTCCCCGCTGAACGACGGAAACTTTGACTGGACTGAC TTGATGACTGG	6
pJBL5819	TGAACTGTATACATTCCCCGCAGAGTGAGTAGATAGGATTGGAGATAGG AGCGGGATATTG	31
pJBL5821	TGAACTGTATACATTCCCCGCAAGGATTAAGAACATTGGGCAAGGATAGA TGAGATTATGA	48
pJBL5823	TGAACTGTATACATTCCCCGCAAGGACAAGACAGGACAGGACATAAAGA TAGGACAGGGCA	23
pJBL5825	TGAACTGTATACATTCCCCGCAAATGGCAACCGGCTTGATAAACTCTATG GACTTCGTATG	86
pJBL5827	TGAACTGTATACATTCCCCGCAAAGGTATCGTGGTCTATTTCGTCGTTGT CATTGTTATTC	46
pJBL5829	TGAACTGTATACATTCCCCGCAGTAGAGATGAGAATAGATAGGTAAGGTA AGGATGGATGA	52
pJBL5831	TGAACTGTATACATTCCCCGCACGAGAGCATAGAGATAGATAGATAGAG	53

	GTGAGTTGAGAG	
pJBL5833	TGAACTGTATACATTCCCCGCAGAGATAAGTAGATGGAAATAGATGAGGACGATGATGAGG	21
pJBL5835	TGAACTGTATACATTCCCCGCACGAGACAAGGCAGAAGTTAATGGAATAAGGGTGATAAGA	17
pJBL5837	TGAACTGTATACATTCCCCGCATAAGTGCTTGTCGTTTCTGTTTCTGCTGTAATTCTCGTC	68
pJBL5839	TGAACTGTATACATTCCCCGCAGGTTATGAATTCTTGCTTATCTTTGCTTATCTATGTTTC	84
pJBL5841	TGAACTGTATACATTCCCCGCAGATGAGGTGAGATAGGATGAGGATAAGTGAGGTGGCGAG	39
pJBL5843	TGAACTGTATACATTCCCCGCAGATGGGAATATGAGATGATGAGATGAAATGAGAGCGAGG	24
pJBL5845	TGAACTGTATACATTCCCCGCAACGAGATAGATGAATGAGATGAGAATGAGATGAGATGGG	13
pJBL5847	TGAACTGTATACATTCCCCGCATAGTTCGGTATCGTATTCTGTCTTGTTCTTATTCTTGTC	36
pJBL5849	TGAACTGTATACATTCCCCGCAGTATGTATGTTATGTCCGTCGTTTCTGTTTCTATTTGTC	72
pJBL5851	TGAACTGTATACATTCCCCGCAAGAATAGATGGGTAGAGATAGATGATGAAATGAAATGAG	26
pJBL5853	TGAACTGTATACATTCCCCGCAGAGACGAAGATAGGGATTGAGGATTGATGAGATGAGGGC	80
pJBL5855	TGAACTGTATACATTCCCCGCAGGACAAGAATAGAATAGGTAGAGGTAAGATGAGAGTGGG	54
pJBL5857	TGAACTGTATACATTCCCCGCAAGAATGCAGAGATAAGTAAGATATGAAATGGATAGGGAC	79
pJBL5861	TGAACTGTATACATTCCCCGCAAAGTCCAGTCTATCGTATTCATTTGCGTTCTTATCATTC	71
pJBL5865	TGAACTGTATACATTCCCCGCAGGATGGACGACTATTGACTTTACTTATGACTGGCTTATG	42
pJBL5867	TGAACTGTATACATTCCCCGCAAGACGAGATAAAGATGGATGAGATGGAGATTGAAGATTG	30
pJBL5869	TGAACTGTATACATTCCCCGCAGGATGAGATGAGAACGATAGAGATGCAAAGGTAAGATGG	1
pJBL5871	TGAACTGTATACATTCCCCGCAGAGACAGGTAGAGATGAAGATTAGAGGCGGATAGATTTG	91
pJBL5875	TGAACTGTATACATTCCCCGCAAAGCAATAGGCAAGGCAGCAGTCGTGAGAGTTAAGGTGG	38
pJBL5877	TGAACTGTATACATTCCCCGCAAAGATGATGATATTGGAGGCTGCTTATTCGTGACTGTGA	45
pJBL5879	TGAACTGTATACATTCCCCGCAACAGAGATAGATAGATGGTAAGGATAGAGGCGAGGTGAG	67
pJBL5881	TGAACTGTATACATTCCCCGCAGAGATAGAAATGGACAATAGGAGCAAGATAAGGATGAGG	16
pJBL5883	TGAACTGTATACATTCCCCGCACGATGAATGGTAAAGATGAAGATGAAGATGAAGGTGAGG	47
pJBL5885	TGAACTGTATACATTCCCCGCAATACTACCATCCCTACCCTACCTTGTCTCCGCTCTATCC	97
pJBL5887	TGAACTGTATACATTCCCCGCATCAAAGTCAAGTTCCCAAGTTCCCATTCATTCATATTCC	100
pJBL5889	TGAACTGTATACATTCCCCGCAACGAACGGGAACTGAGGACTTACTGGACTTTGACTACTG	89
pJBL5891	TGAACTGTATACATTCCCCGCATGGTCCCGTTATTCTATTGTCTTTGCTTGCTTGTTATTG	33
pJBL5893	TGAACTGTATACATTCCCCGCAAGGACAGTAAGAATAGATAAGTAGAGGTGAGTGGAGATG	78
pJBL5895	TGAACTGTATACATTCCCCGCAAAGTGGACGAGATAGGTAGCGGGATTAAGAATTGGAGAC	58
pJBL5897	TGAACTGTATACATTCCCCGCAAGGACATATTGAAGATTGAAACTGGTATTGTAAGGCGAG	64
pJBL5899	TGAACTGTATACATTCCCCGCAGACGGAGATAGATGAGACGAATGGAGATGAATAAGAGCG	41
pJBL5901	TGAACTGTATACATTCCCCGCAAAGTGAGATGATAATGAGATAAGAATATGGAGATGGAGG	2
pJBL4145	TGAACTGTATACATTCCCCGCAAAGACAAAGACGATAAGATAATGAAATGAAACGCGAAAG	62

pJBL4147	TGAACTGTATACATTCCCCGCTAAGAACAAGATAATGAAATGAGATGATA AAGCAAATAGG	59
pJBL4149	TGAACTGTATACATTCCCCGCAGTGAGGATGAGATGAGAGTGATAGATG AGCGAGATAGAG	40
pJBL4151	TGAACTGTATACATTCCCCGCAGTAGAAGTAGTGAGTAAGTGAGGTAGAA ATGGGTAGGAG	61
pJBL4153	TGAACTGTATACATTCCCCGCACCGTGTAAAGCAGCCGGTTTCGAACAA GTGGAGTAGGCG	85
pJBL2807	TGAACTGTATACATTCCCCGCTGCTCCAACATTTATACAACTAATTAAAAC AATTCACTGTAAAAACT	WT and 28 and length variant used in SI 1
pJBL2808	TGAaCTGTATACATTCCCCGCTGCTCCAACATTTATACAACTAATTAAAAC AATTCACTGTAAAAACTTTTCCTAGAC	Length variant used in SI 1
pJBL2806	TGAACTGTATACATTCCCCGCTGCTCCAACATTTATACAACTAATTAAAAC AATTCAC	Length variant used in SI 1
pJBL2805	TGAACTGTATACATTCCCCGCTGCTCCAACATTTATACAACTAATTAA	Length variant used in SI 1
pJBL2804	TGAACTGTATACATTCCCCGCTGCTCCAACATTTATAC	Length variant used in SI 1
pJBL2115	TGAACTGTATACATTCCCCGCTGCTCCAACATT	Length variant used in SI 1
pJBL5926	TGAACTGTATACATTCCCCGC	Length variant used in SI 1
pJBL4135	TGCTCCAACATTTATACAACTAATTAAAACAATTCACTGTAAAAACT	Length variant used in SI 1
pJBL4134	CCGCTGCTCCAACATTTATACAACTAATTAAAACAATTCACTGTAAAAACT	Length variant used in SI 1
pJBL4133	TCCCCGCTGCTCCAACATTTATACAACTAATTAAAACAATTCACTGTAAAA ACT	Length variant used in SI 1
pJBL4132	CATTCCCCGCTGCTCCAACATTTATACAACTAATTAAAACAATTCACTGTA AAAACT	Length variant used in SI 1
pJBL4131	CATTCCCCGCTGCTCCAACATTTATACAACTAATTAAAACAATTCACTGTA AAAACT	Length variant used in SI 1
pJBL4130	TGTATACATTCCCCGCTGCTCCAACATTTATACAACTAATTAAAACAATTC ACTGTAAAAACT	Length variant used in SI 1
pJBL6071	TTATTTTGCTTCCGGCAAGCAAATCAGAGACAGGACAGGACAAATTGGA GACACAGAGATAGAG	RibA 1
pJBL6070	TTATTTTGCTTCCGGCAAGCAAATAGGGATGAGAGTAGAGTGGATGAATG AGATGGGATGGGAG	RibA 2
pJBL6072	TTATTTTGCTTCCGGCAAGCAAATCTTAAGTTCTTGTAGTCCGTCCCGTT GTCCCGTAGTATGC	RibA 3
pJBL6073	TTATTTTGCTTCCGGCAAGCAAATACGCTTACGCTACCTACCTACCCTAC ACCTTGACCCTAAC	RibA 4
pJBL6074	TTATTTTGCTTCCGGCAAGCAAATGCCGTCGCCCTATGTTCTGTTCTTAT CGTTTACTTGTCTC	RibA 5
pJBL5576	TGAACTGTATACATTCCCCGCTAACTCCATTCCATC	Input B AND gate
pJBL5577	GATGGAATGGAGTTAAGGATAAGAGTAAGTGAGAGTAGGTAGAGATTGA GGATGG	Input A AND gate

Supplementary Table 6. Strains used in this study. Strains containing genomic insertions were created using the clonetegration platform[7] to integrate the inserts using the HK022 plasmid into the *attB* site of the *E. coli* genome. Successful integrations were identified by antibiotic selection and colony PCR according to the published protocol[7].

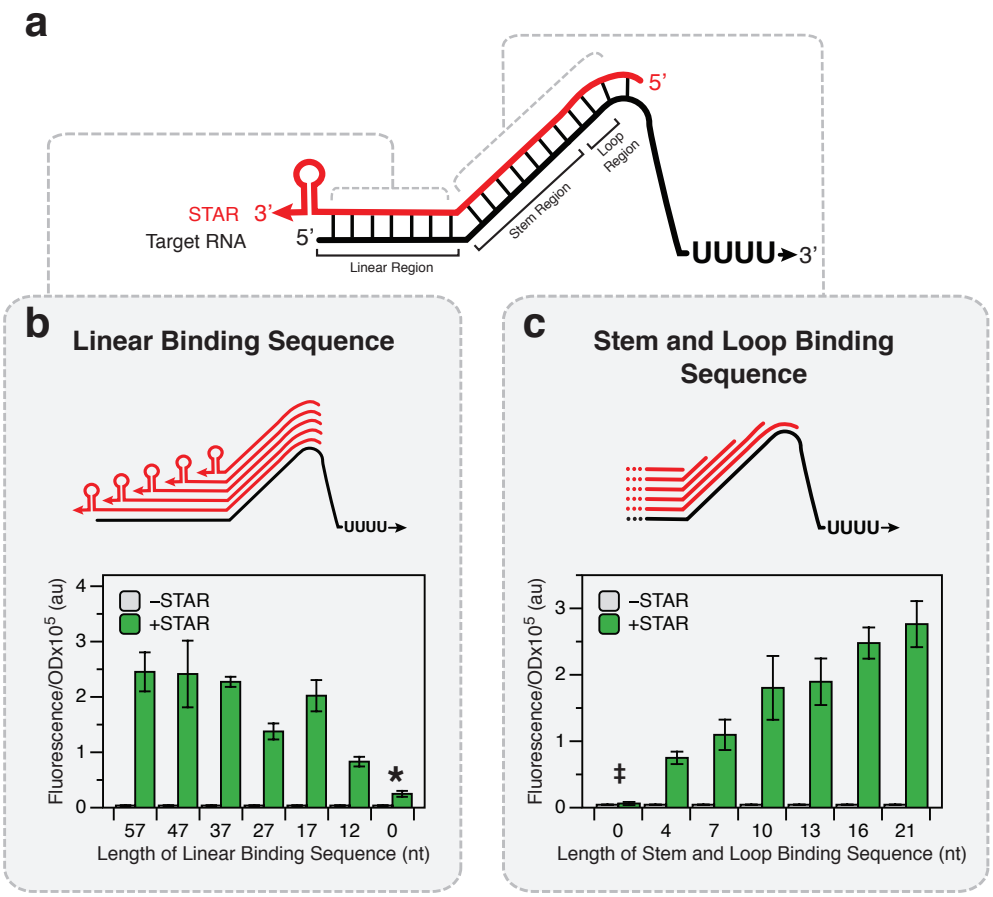
Strain	Strain Genotype	Genomic Insertion
E. coli TG1	K-12 *supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5, ($r_K^-m_K^-$)* F' [*traD36 proAB$^+$ lacI^q lacZΔM15]*	N/A
E. coli TG1 target variant 5 *sfgfp*	K-12 *supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5, ($r_K^-m_K^-$)* F' [*traD36 proAB$^+$ lacI^q lacZΔM15] attB::target variant 5 sfgfp CmR*	*attB::target variant 5 sfgfp(* Promoter 1 – Target 5 – RBS 1 – sfGFP – TrrnB – CmR)
E. coli BW25113	F$^-$, *Δ* (araD-araB)567, lacZ4787(del)::rrnB-3, LAM$^-$, rph-1, *Δ* (rhaD-rhaB)568, hsdR514	N/A
E. coli BW25113 Δ*cheZ*	F$^-$, *Δ* (araD-araB)567, lacZ4787(del)::rrnB-3, LAM$^-$, rph-1, *Δ* (rhaD-rhaB)568, hsdR514, *ΔcheZ734::kan*	N/A
E. coli BW25113 Δ*cheZ* target variant 5 *cheZ*	F$^-$, *Δ* (araD-araB)567, lacZ4787(del)::rrnB-3, LAM$^-$, rph-1, *Δ* (rhaD-rhaB)568, hsdR514, *ΔcheZ734::kan, attB::target variant 5 cheZ CmR*	*attB::target variant 5 cheZ* (Promoter 1 – Target 5 – RBS 1 – CheZ – TrrnB – CmR)
E. coli DH5 alpha pir	F-, *Δ(argF-lac)169*, *ϕ* 80d*lacZ58*(M15), *ΔphoA8*, *glnX44*(AS), *λ*$^-$, *deoR481*, *rfbC1*, *gyrA96*(NalR), *recA1*, *endA1*, *thiE1*, *hsdR17, Δ uidA3::pir*	N/A

Supplementary Table 7: Primers used for reverse transcription and quantitative PCR

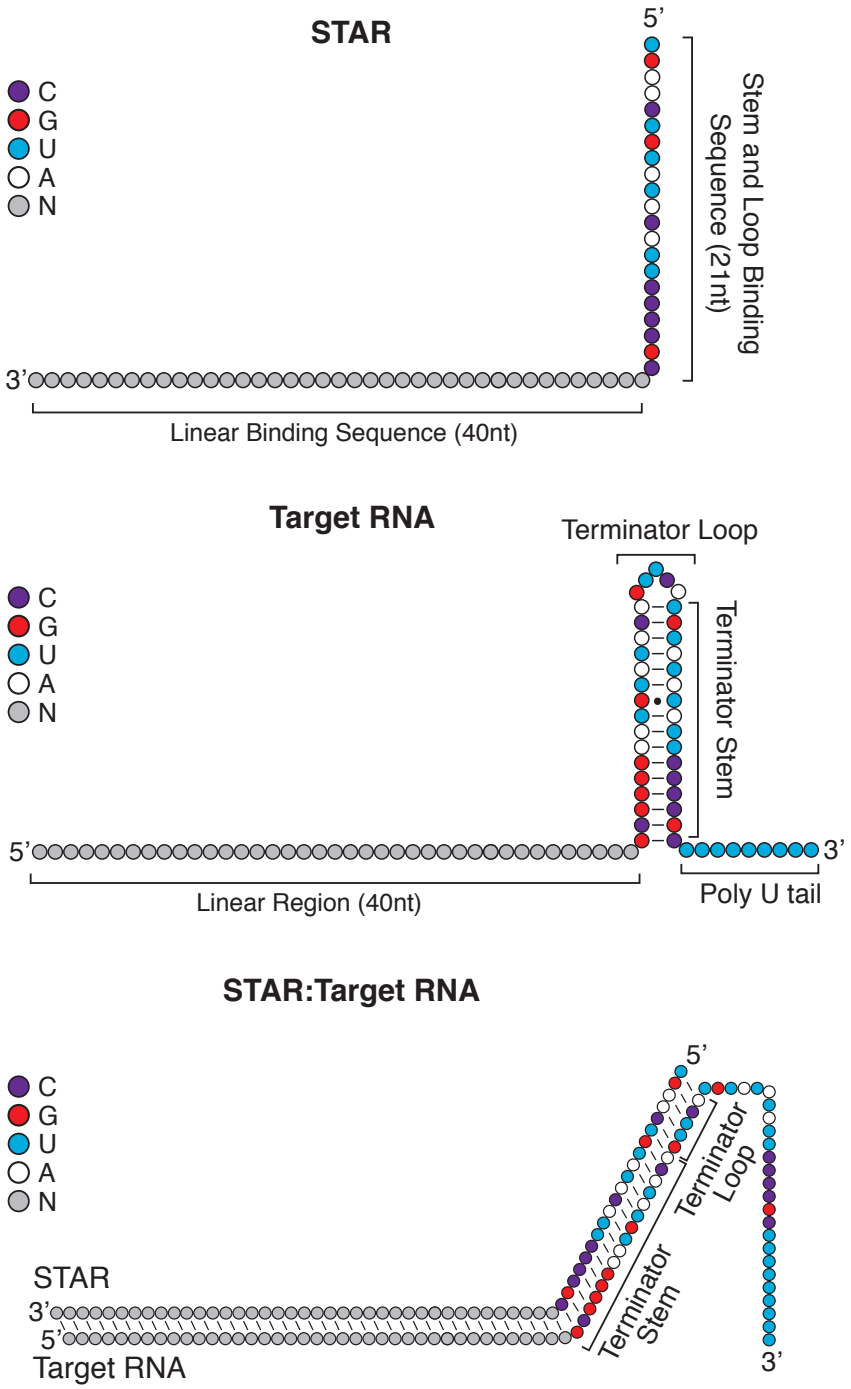
Primer Name	Sequence (5' to 3')
RT.sfGFP	TTATTTGTAGAGCTCATCCATG
sfGFP.Fwd	CACTGGAGTTGTCCCAATTCT
sfGFP.Rev	TCCGTTTGTAGCATCACCTTC

Supplementary Table 8: Sequences of toehold switch and trigger used. The best performing toehold switch and trigger (referred to as forward-engineered 1) were derived from the original paper by Green *et al*.[8]

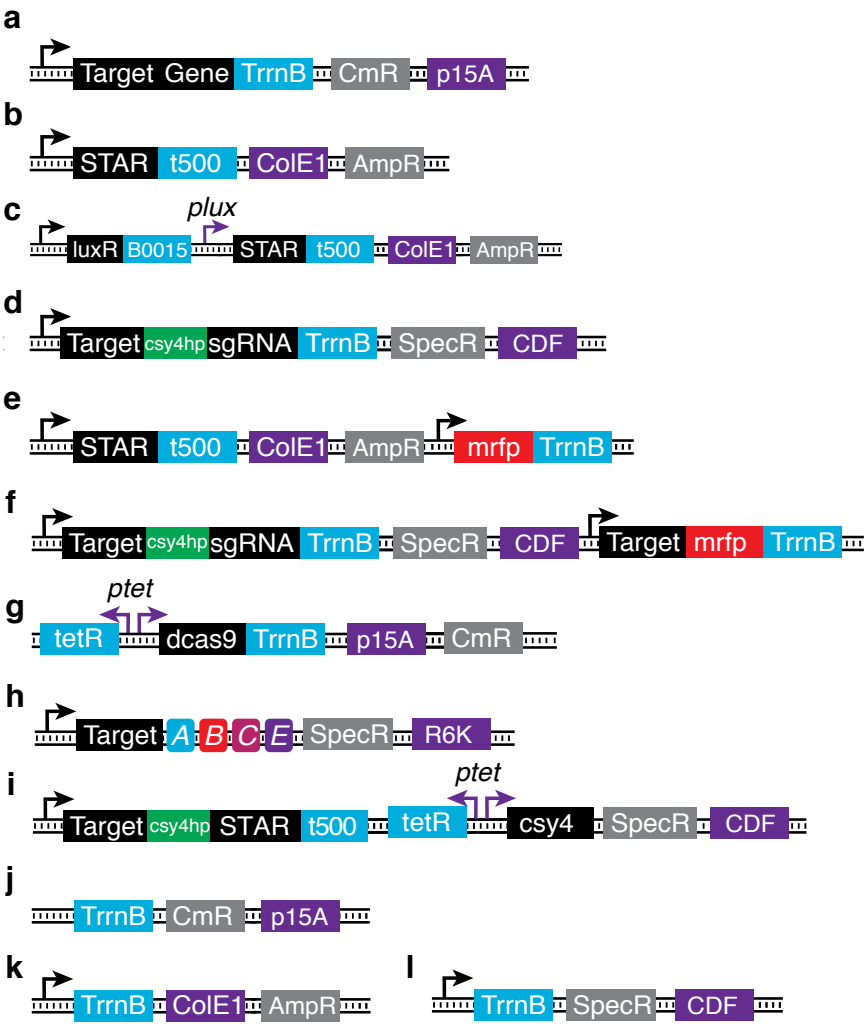
Name	Sequence (5' to 3')
Toehold Switch 1: Switch 1 - linker	GGGTCTTATCTTATCTATCTCGTTTATCCCT GCATACAGAAACAGAGGAGATATGCAATGATAAACGAG AACCTGGCGGCAGCGCAAAAG
Toehold Trigger 1: Stability Hairpin -Trigger 1	GGGACTGACTATTCTGTGCAATAGTCAGTAAA GCAGGGATAAACGAGATAGATAAGATAAGATAG



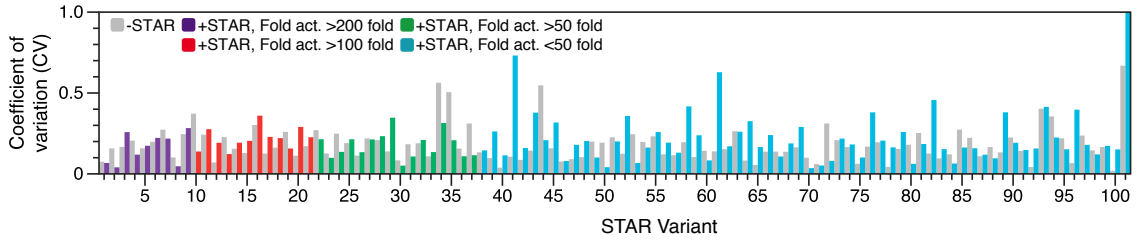
Supplementary Figure 1. Determining a STAR design motif. (**a**) Schematic of a STAR and target RNA complex with the different interaction regions annotated. Schematics and fluorescence characterization of STAR variants that were used to determine optimal lengths of (**b**) the linear binding sequence and (**c**) the stem and loop binding sequence of the AD1 STAR[9]. STAR variants were created by truncating either the (**b**) 3' or (**c**) 5' end of the STAR while the target RNA was kept constant. Characterization revealed the optimal lengths were 21 nucleotides (nt) for the stem and loop binding sequence and ~40 nt for the linear binding sequence. In addition, it was observed that neither the stem and loop binding sequence itself (0 nt in (**b**) indicated by *) or the linear binding sequence itself (0 nt in (**c**) indicated by ‡) of the STAR were sufficient to appreciably activate transcription. This reveals a design motif to create orthogonal and functionally diverse variants by varying the linear region of target/STARs while maintaining a constant stem and loop region. Fluorescence characterization (measured in units of fluorescence/optical density [OD] at 600 nm) was performed on *E. coli* cells transformed with the AD1 target DNA plasmid in the absence (-STAR) and presence (+STAR) of a DNA plasmid encoding a cognate STAR length variant. Data represents mean values of n = 9 biological replicas ± s.d.



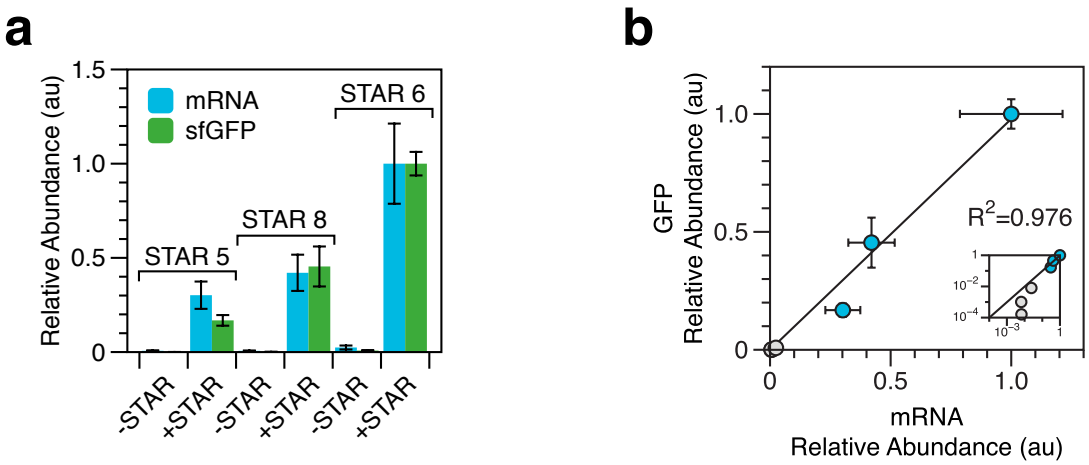
Supplementary Figure 2. Schematic of the STAR design motif used in NUPACK. Schematic of the sequence and structure constraints for the STAR, target RNA and STAR-target RNA complex used in the NUPACK design algorithm[10, 11]. Nucleotides colored according to identity with N representing an unconstrained nucleotide that is designed by NUPACK. See **Supplementary Note 1** for a description of the NUPACK script used.



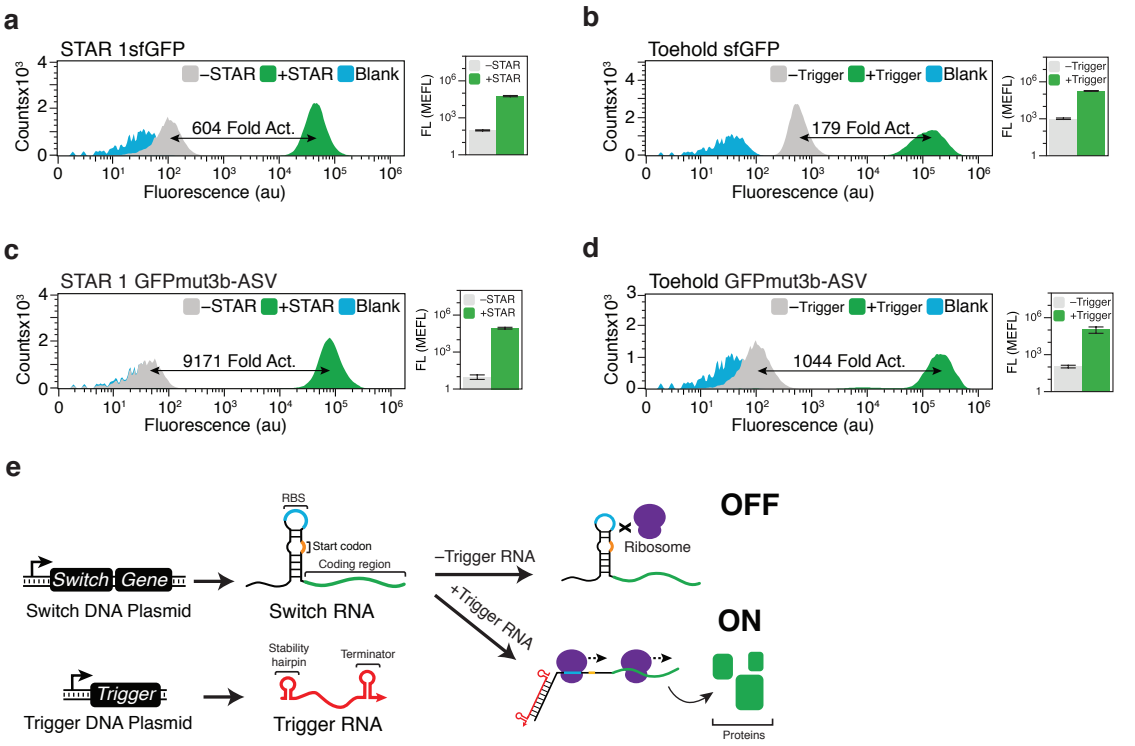
Supplementary Figure 3. Schematic of representative DNA plasmid maps used in this study. (**a**) Target RNA expressing plasmid, (**b**) STAR expressing plasmid, (**c**) AHL inducible STAR expressing plasmid, (**d**) STAR regulated sgRNA plasmid, (**e**) STAR and mRFP expressing plasmid, (**f**) STAR regulated sgRNA and mRFP plasmid, (**g**) dCas9 expressing plasmid, (**h**) STAR regulated deoxyviolacein plasmid, (**i**) Stage 2 activation-activation cascade expressing plasmid, (**j**) no target RNA control plasmid, (**k**) no STAR control plasmid and (**l**) no sgRNA/no Target control plasmid. Constitutive promoters are colored black and inducible promoters are colored purple and labelled accordingly.



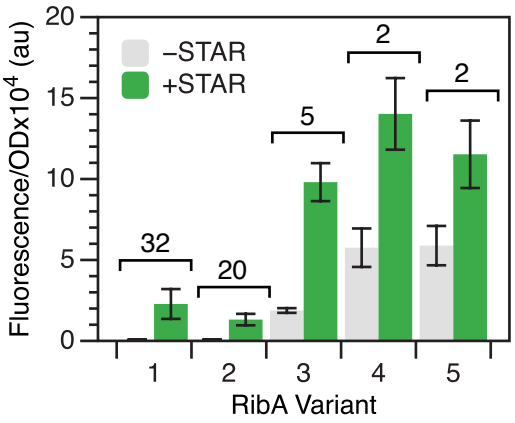
Supplementary Figure 4. Coefficient of variation for fluorescence characterization of STAR variants. Coefficient of variation (CV) was determined by calculating the ratio of the standard deviation and the mean of fluorescence measurements for the STAR/target variants. Data is derived from fluorescence characterization described in **Figure 1b** for both in the absence (–STAR) and the presence (+STAR) of cognate STAR expression plasmids. The y-axis was limited to 1 to aid interpretation. The CV for variant 101 in the +STAR condition was 1.93.



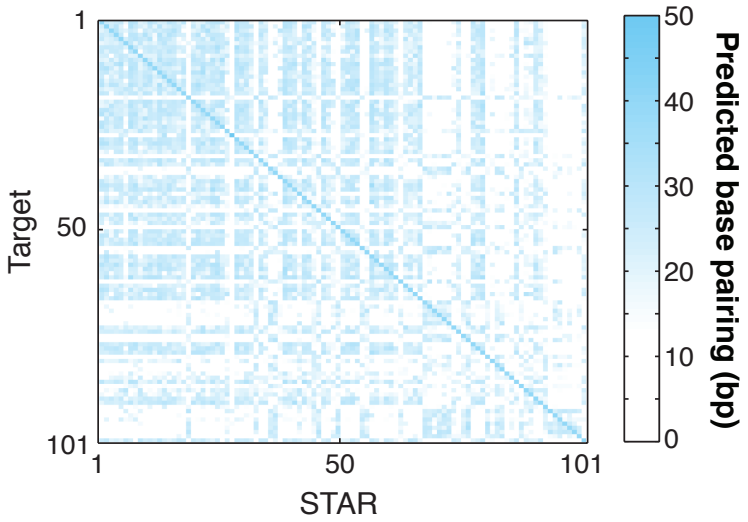
Supplementary Figure 5. Comparison of reverse transcription quantitative PCR (RT-qPCR) and fluorescence characterization of STAR variants. Relative abundance of sfGFP mRNA (mRNA) and protein expression (sfGFP) for three STAR/target variants (**a**, **b**). RT-qPCR (measuring relative abundance of sfGFP mRNA) and fluorescence characterization (measuring relative abundance of sfGFP protein in units of fluorescence/optical density [OD] at 600 nm) was performed on *E. coli* cells transformed with different target DNA plasmids in the absence (-STAR) and presence (+STAR) of a DNA plasmid encoding a cognate STAR. (**b**) Correlation of determination (R^2) between RT-qPCR and fluorescence characterization is indicated. Data for each type of measurement were normalized to 1 for the +STAR condition of STAR variant 6 and error propagated. Fluorescence data represents mean values of n = 9 biological replicas ± s.d. RT-qPCR data represents mean values of n = 3 biological replicas each quantified with n = 3 technical replicas ± s.d.



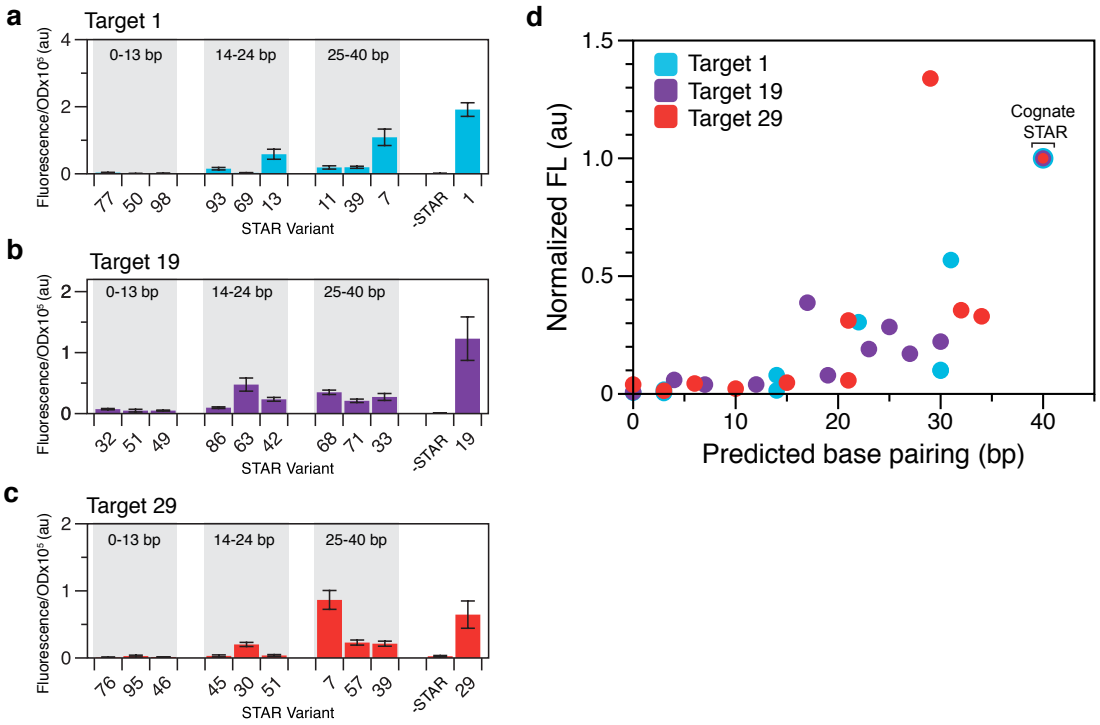
Supplementary Figure 6. Comparison of a STAR and a toehold translational activator. Fluorescence characterization of the best-performing STAR and the best performing toehold translational activator from Green *et al*.[8] Fluorescence characterization performed with both (**a**, **b**) sfGFP and (**c**, **d**) GFPmut3b-ASV. Flow cytometry histograms of a representative biological replicate shown on the left of each panel and graph of mean fluorescence of biological replicates shown on the right of each panel. (**e**) Toehold switches function by designing a switch RNA to form a hairpin structure around the ribosome binding site (RBS) to repress ribosome binding and translation initiation. Activation is achieved by addition of a trigger RNA designed to disrupt hairpin formation and allow translation initiation. The best performing toehold switch (designated switch 1 in Green *et al*.) was chosen for characterization within the experimental setup described for STARs. We note several major differences in the characterization experiment performed here and that performed in Green *et al*. including: using *E. coli* RNA polymerase (RNAP) to express trigger and switch RNA (originally T7 polymerase), use of *E. coli* RNAP transcriptional terminators, different DNA plasmid back bones, using TG1 *E. coli* strain (originally BL21 STAR DE3) and M9 minimal media (originally LB). Data were collected by flow cytometry. Fluorescence characterization (measured in units of arbitrary fluorescence [au] or units of Molecules of Equivalent Fluorescein [MEFL]) was performed on *E. coli* cells transformed with different target DNA plasmids in the absence (–STAR/–Trigger) and presence (+STAR/+Trigger) of a DNA plasmid encoding cognate STAR/Trigger, compared to the autofluorescence of *E. coli* cells transformed with control plasmids (Blank). Representative flow cytometry histograms of n = 1 biological replicas and bar graphs of at least n = 7 biological replicas ± s.d.



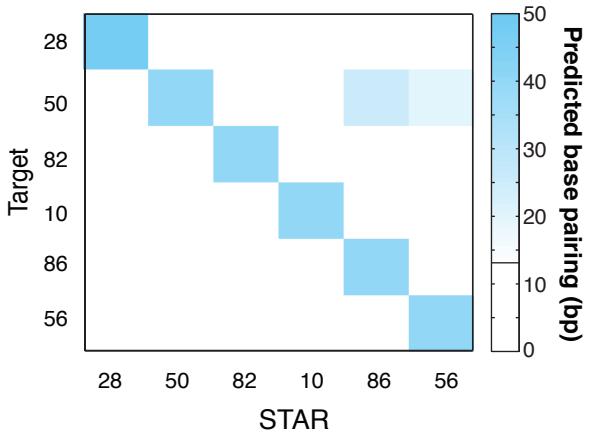
Supplementary Figure 7. Experimental characterization of ribA STARs. Characterization of five STAR-target RNAs utilizing the terminator from the *E. coli* ribA gene. Fluorescence characterization (measured in units of fluorescence/optical density [OD] at 600 nm) was performed with *E. coli* cells transformed with DNA target plasmids in the absence (–STAR) and presence (+STAR) of a DNA plasmid encoding cognate STAR. Data represents mean values of n = 9 biological replicas ± s.d.



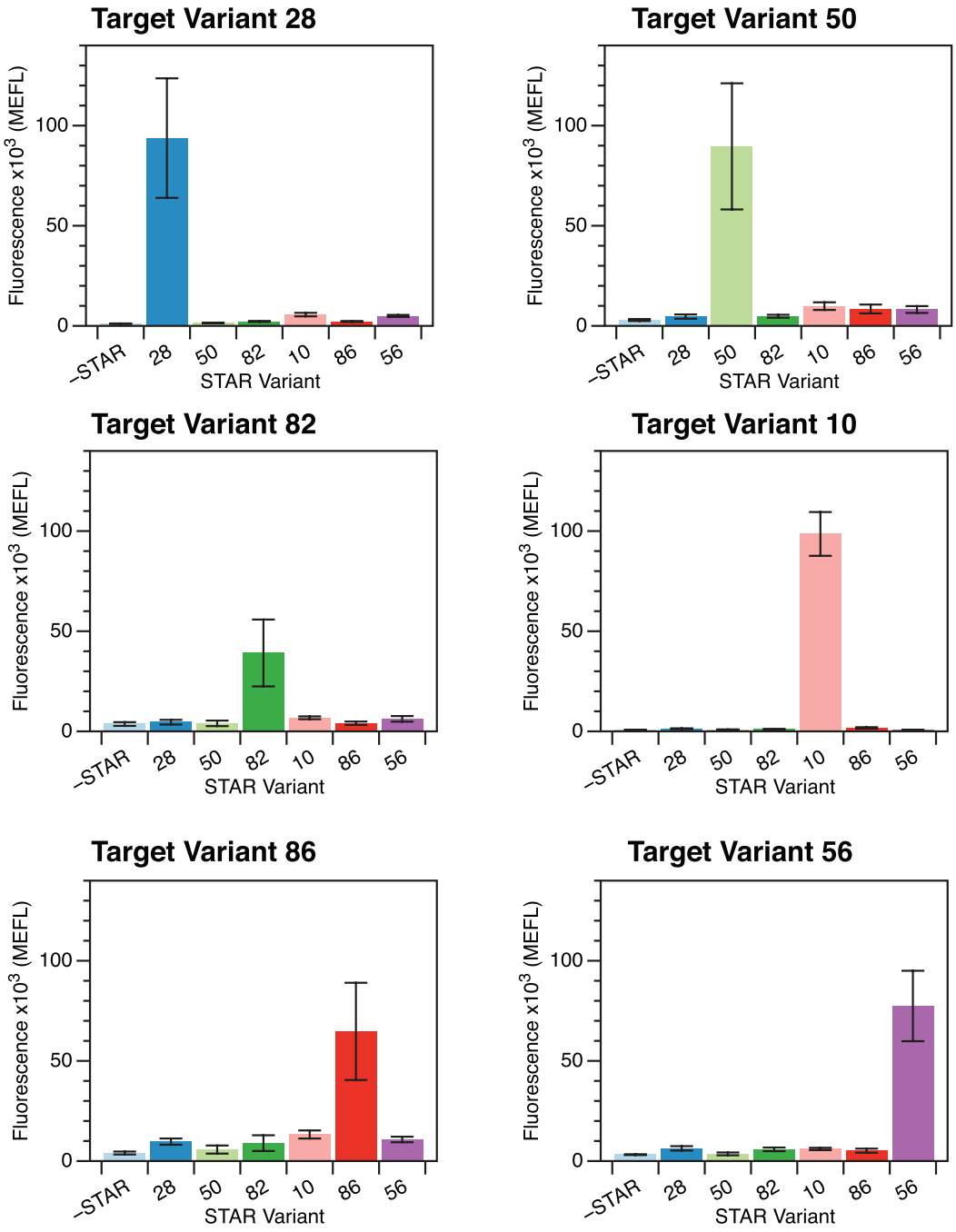
Supplementary Figure 8. Computational prediction of base pairing between STAR and target RNA variants. Computationally predicted base pairing (bp) between the STAR and target RNA variants using NUPACK as described in **Supplementary Note 2**. The matrix shows the predicted pairing for each combination of the 101 STAR/target RNA variants (10,201 combinations).



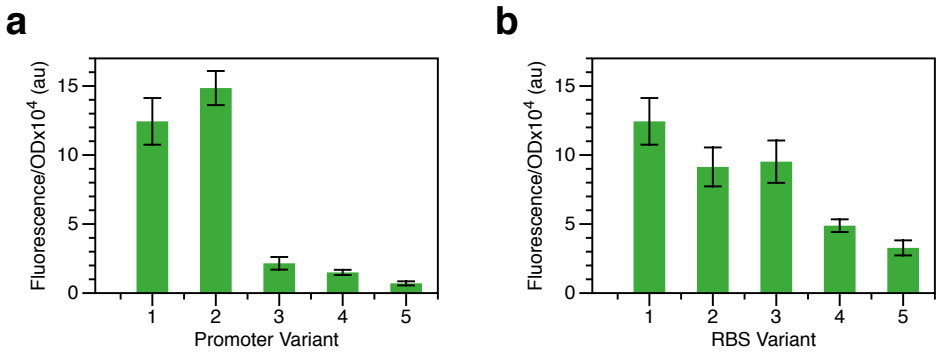
Supplementary Figure 9. Determining the relationship between predicted STAR-target RNA base pairing and orthogonality. (**a-c**) Fluorescence characterization of three target RNAs in combination with 9 non-cognate STARs that were predicted to form between 0-13 base pairs (bp), 14-24 bp or 25-40 bp by the NUPACK analysis algorithm as described in **Supplementary Note 3**. For each target RNA, fluorescence characterization was performed in the absence (-STAR) and presence of a DNA plasmid encoding a STAR variant (indicted below graph) (cognate STAR shown in right most bar in **a-c**). (**d**) Normalized fluorescence of non-cognate and cognate STARs plotted against predicted base pairing. Fluorescence was normalized to the fluorescence value for the cognate STAR. Data represents mean values of *n* = 9 biological replicas ± s.d.



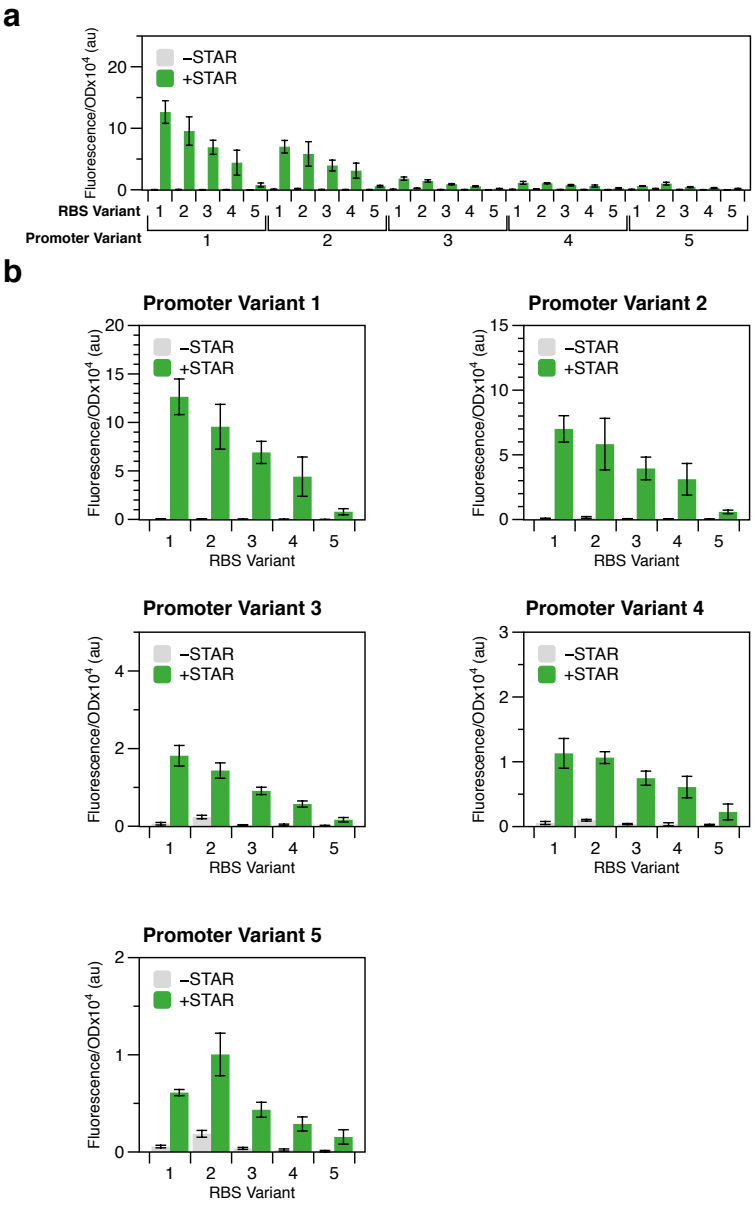
Supplementary Figure 10. Computational prediction of STAR orthogonality. A computationally predicted set of orthogonal STARs was identified using an algorithm described in **Supplementary Note 3**. In this algorithm, STAR/target RNAs were predicted to be orthogonal if base pairing between the linear region and linear binding sequence of a target and STAR was less than 13 bp for non-cognate pairs in one of the two combinations (i.e. either STAR 1/target 2 or STAR 2/ target 1). The matrix shows the predicted pairing for each combination of a specific set of STAR/target RNA variants. Less than 13 bp of interaction are colored white and 50 bp of interaction are colored blue. These predictions were validated experimentally through gene expression measurements in *E. coli* cells shown in **Fig. 1d**.



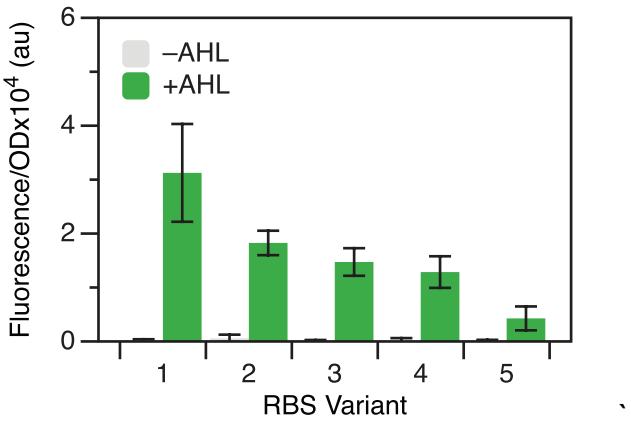
Supplementary Figure 11. Experimental characterization of an orthogonal STAR library. Orthogonality of a 6x6 library of target RNA and STAR variants. Fluorescence characterization (in units of Molecules of Equivalent Fluorescein [MEFL]) was performed on *E. coli* cells transformed with different target DNA plasmids (labeled above each panel) in the absence (-STAR) and presence of a DNA plasmid encoding a cognate or non-cognate STAR variant (shown on x-axis of each panel). Data were measured with flow cytometry. Data represents mean values of at least n = 7 biological replicas ± s.d.



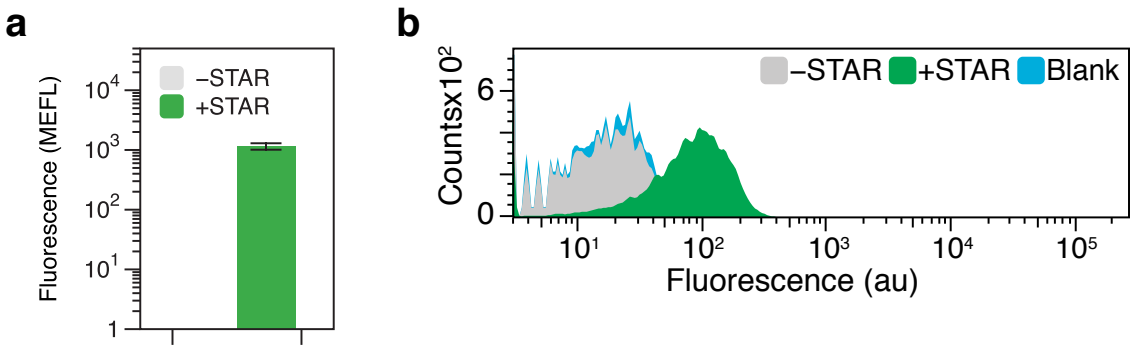
Supplementary Figure 12. Variable strength promoter and RBS library. Characterization of five different strength promoters and ribosome binding sites (RBS) variants (see **Supplementary Table 3** for sequences). Promoter and RBS variants were used to drive expression of a sfGFP containing an RNA stability hairpin derived from a previously published library[6]. (**a**) RBS 1 was used for the promoter library and (**b**) promoter 1 was used in the RBS library. Fluorescence characterization (measured in units of fluorescence/optical density [OD] at 600 nm) was performed on *E. coli* cells transformed with a library of DNA plasmids containing variable strength promoters and RBSs. Data represents mean values of n = 9 biological replicas ± s.d.



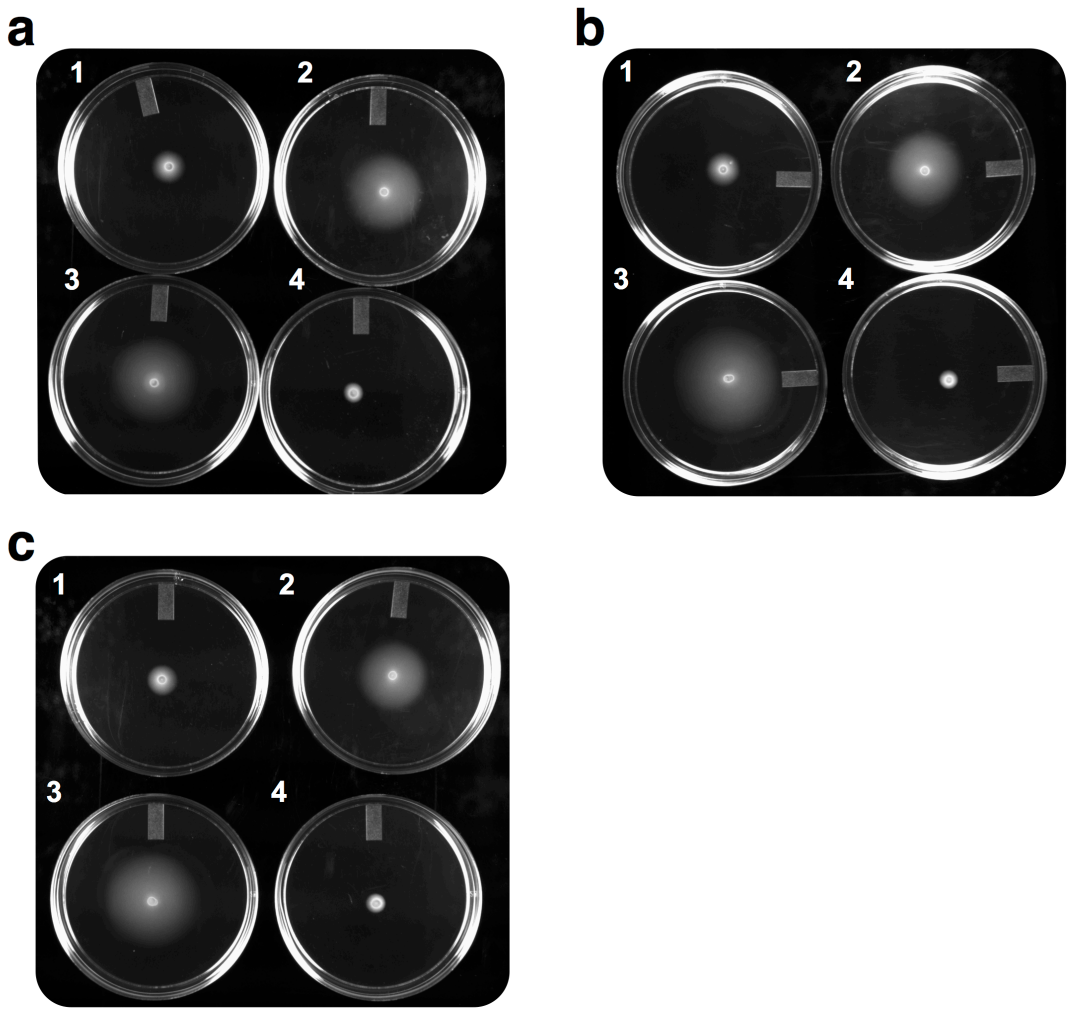
Supplementary Figure 13. Creating switchable promoter and ribosome binding site strength libraries with STARs. Target variant 5 was combined with 5 different strength promoters and ribosome binding sites (RBS) variants (see **Supplementary Table 3** for sequences). Promoters and RBSs variants are numbered 1-5 from strongest to weakest. Fluorescence characterization (measured in units of fluorescence/optical density [OD] at 600 nm) was performed on *E. coli* cells transformed with a library of target DNA plasmids containing variable strength promoters and RBSs in the absence (-STAR) and presence (+STAR) of a DNA plasmid encoding STAR variant 5. (**a**) Full library and (**b**) each promoter variant are shown in panels with RBS variants shown on the x-axis. Data represents mean values of n = 9 biological replicas ± s.d.



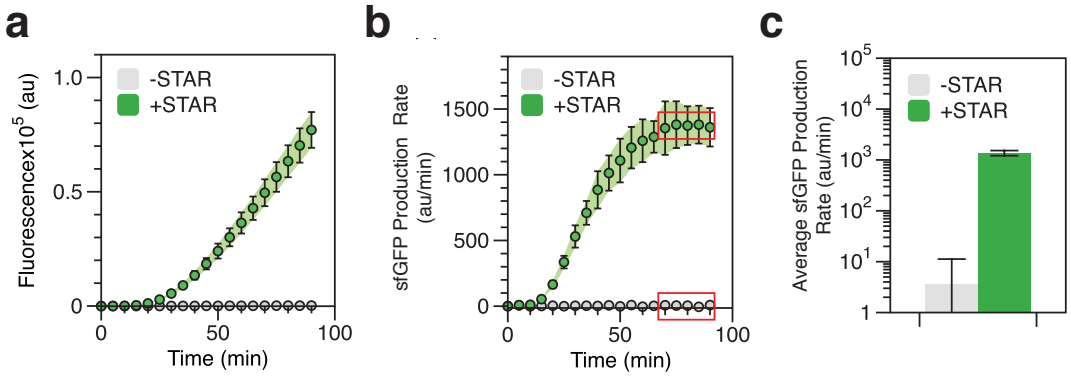
Supplementary Figure 14. Combining the inducible promoter-STAR system with the target RNA RBS strength library. The inducible promoter-STAR system was combined with the target RNA RBS strength library and assayed for function. Fluorescence characterization (measured in units of fluorescence/optical density [OD] at 600 nm) was performed on *E. coli* cells transformed with DNA target-RBS library plasmids in the absence (–AHL) and presence (+AHL) of 100 nM AHL. Data represents mean values of n = 9 biological replicas ± s.d.



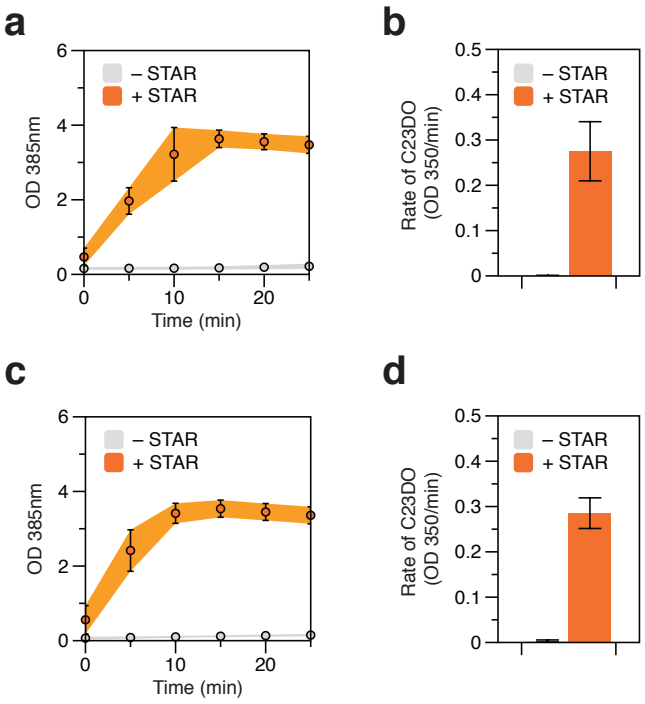
Supplementary Figure 15. STARs regulate sfGFP expression from target expression cassettes integrated into the *E. coli* genome. A STAR variant 5 regulated *sfgfp* gene was integrated into the genome of *E. coli* TG1 cells to create *E. coli* TG1 target variant 5 *sfgfp* (**Supplementary Table 6**). Fluorescence characterization was performed on *E. coli* TG1 target variant 5 *sfgfp* cells in the absence (-STAR) and presence (+STAR) of a DNA plasmid encoding STAR variant 5, and compared to the autofluorescence of *E. coli* cells transformed with control plasmids (Blank). Data were collected by flow cytometry. (**a**) Mean fluorescence (measured in units of Molecules of Equivalent Fluorescein [MEFL]) of at least $n = 7$ biological replicas ± s.d. and (**b**) a representative flow cytometry histogram of $n = 1$ biological replicas (measured in units of arbitrary fluorescence [au]).



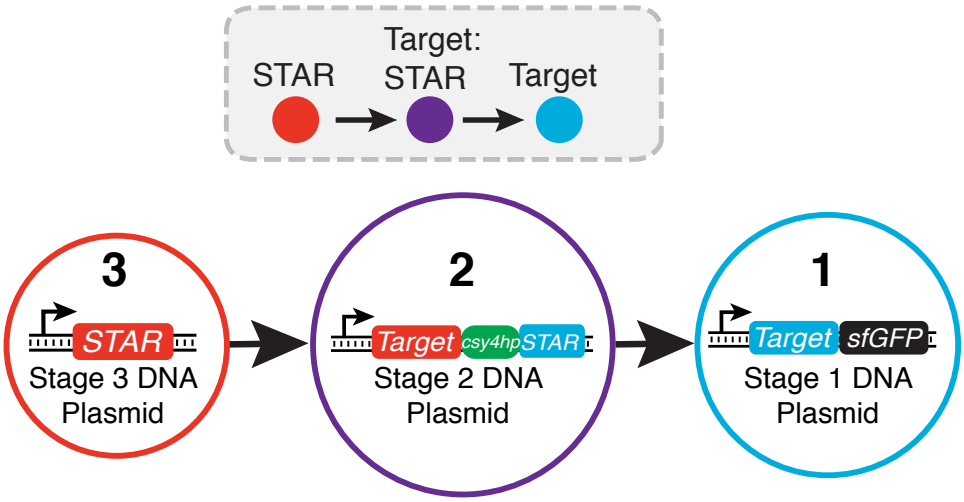
Supplementary Figure 16. Controlling cell motility with STARs. A STAR variant 5 regulated *cheZ* gene was integrated into the genome of *E. coli* BW25113Δ*cheZ* cells to create *E. coli* BW25113Δ*cheZ* target variant 5 *cheZ* (**Supplementary Table 6**). Photographs of semi-solid motility assays of *E. coli* BW25113Δ*cheZ* target variant 5 *cheZ* cells in the absence (1) and presence (2) of a DNA plasmid encoding STAR variant 5. The parent strain *E. coli* BW25113 (3) and *E. coli* BW25113Δ*cheZ* (4) cells transformed with control plasmids were used as motile and non-motile controls. Each photograph show results from $n = 1$ biological replicates performed independently (**a**-**c**).



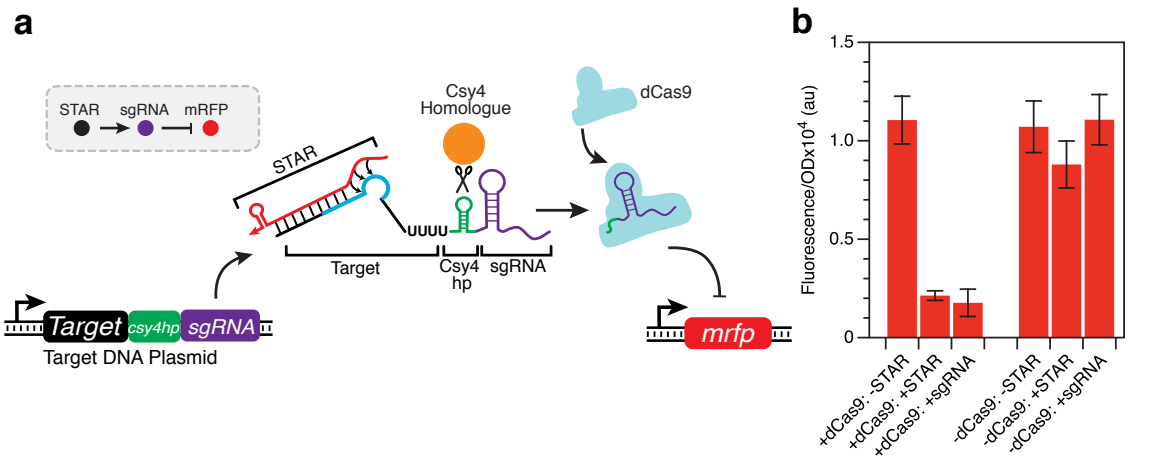
Supplementary Figure 17. Characterization of STARs in cell-free transcription and translation (TX-TL) reactions. Characterization of STAR variant 5 expressing sfGFP in TX-TL reactions. (**a**) Fluorescence characterization (measured in units of arbitrary fluorescence [au]) performed in TX-TL reactions containing 8 nM of target DNA plasmid variant 5 and either 15 nM of a no-STAR control plasmid (-STAR) or 15 nM of a DNA plasmid encoding STAR variant 5 (+STAR). (**b**) The production rate of sfGFP (measured in units of au/minute) was determined by taking the time derivative of (**a**). (**c**) The average sfGFP production rate was determined within the linear phase of sfGFP production rate highlighted in the red box in (**b**). Data represents mean values of *n* = 9 biological replicas ± s.d.



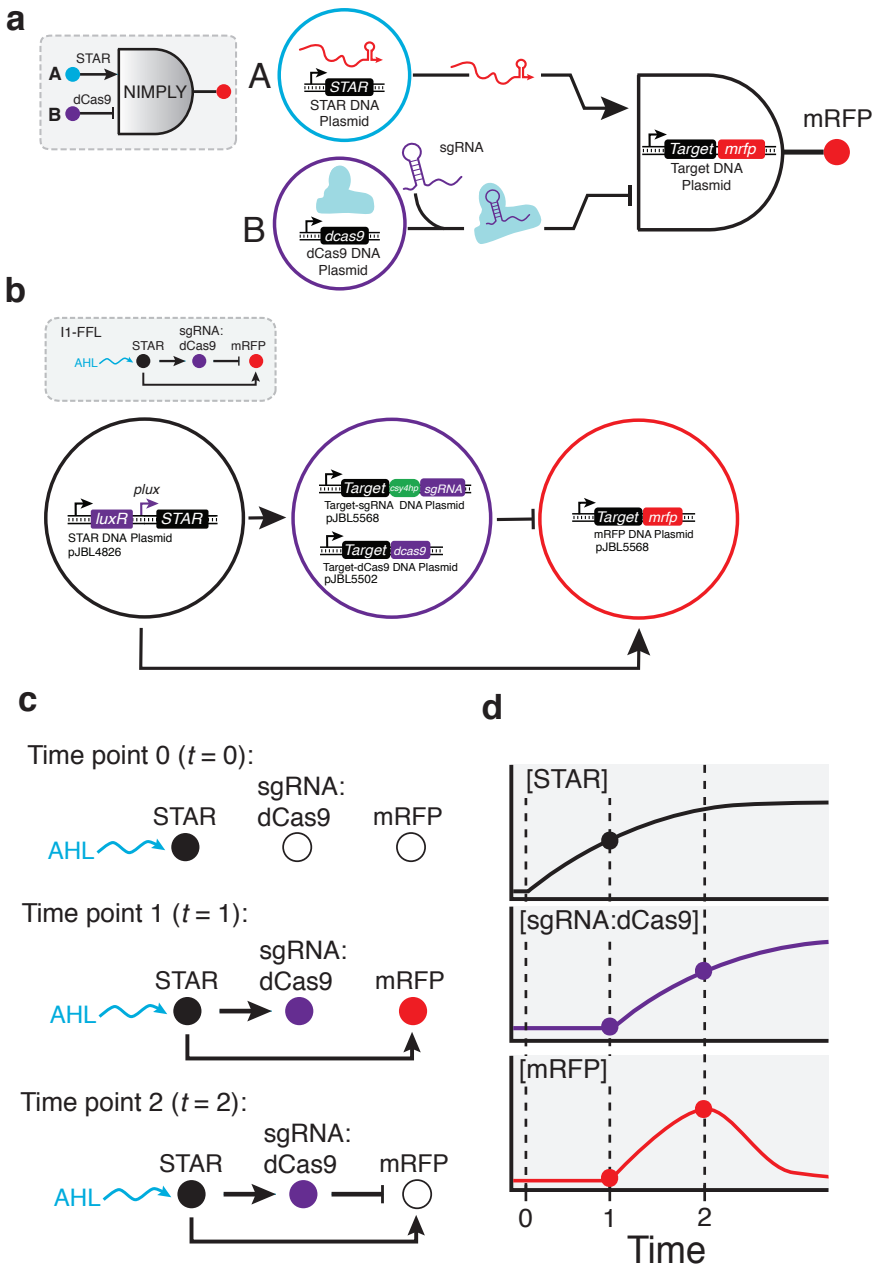
Supplementary Figure 18. Characterization of catechol 2,3-dioxygenase (C23DO) enzyme expression for one-to-one and one-to-many regulation. STAR controlled expression of C23DO in *E. coli* cells for (**a**, **b**) one-to-one and (**c**, **d**) one-to-many regulation. (**a**, **c**) Kinetic spectral characterization (measured in units of OD at 385 nm) was used to determine the (**b**, **d**) rate of C23DO conversion of catechol to 2-hydroxymuconate semialdehyde between 0 and 10 minutes. C23DO expression characterization was performed on *E. coli* cells transformed with either (**a**, **b**) a DNA plasmid encoding target RNA regulated C23DO alone or (**c**, **d**) DNA plasmids encoding target RNA regulated C23DO, mRFP and sfGFP simultaneously. Data represents mean values of n = 9 biological replicas ± s.d.



Supplementary Figure 19. Schematic of the STAR activation-activation cascade. The activation-activation cascade is composed of three stages. Stage 3 is a STAR that activates its cognate target RNA on stage 2. This target RNA is transcriptionally fused to a Csy4 hairpin (Csy4 hp)[3] and an orthogonal STAR. A heterologously expressed Csy4 gene cleaves[3] the Csy4 hp, releasing the orthogonal STAR from the target RNA, allowing it to activate its cognate target RNA on stage 1 that is transcriptionally fused to an sfGFP gene. sfGFP should only be expressed in the presence of the full cascade.

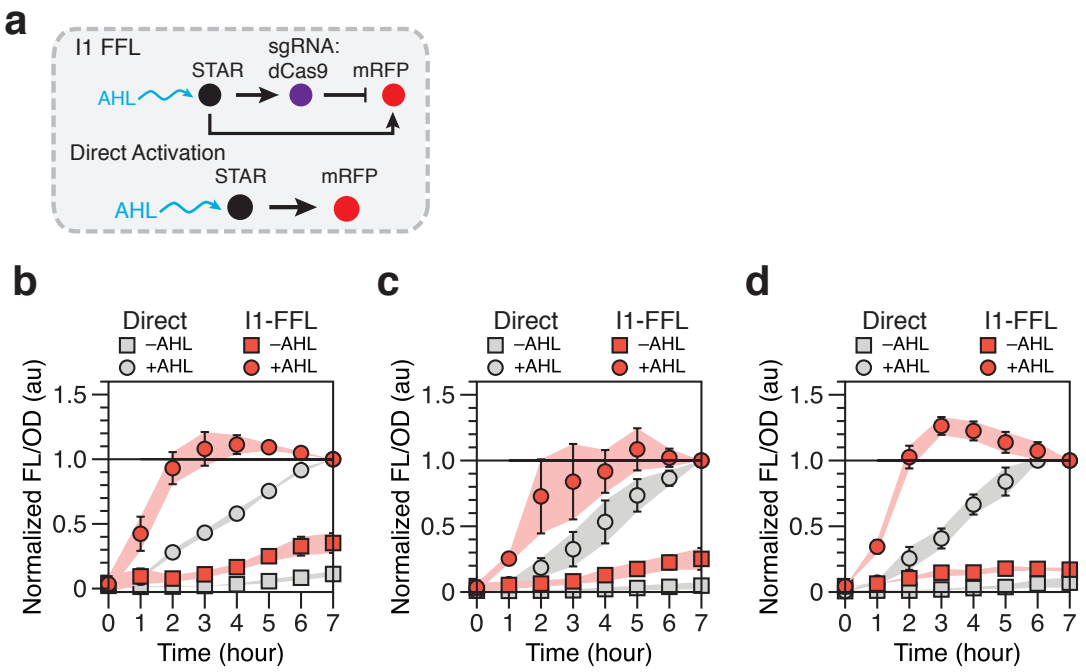


Supplementary Figure 20. Characterization of STAR regulated CRISPR interference (CRISPRi). (**a**) Schematic of STAR regulated CRISPRi. The STAR regulates expression of a target RNA:Csy4 hairpin:single guide RNA (sgRNA) transcriptional fusion. A Csy4 homolog endogenous to *E. coli* can cleave the Csy4 hairpin (Csy4 hp)[3], releasing the sgRNA to form a complex with a catalytically dead mutant of the Cas9 protein (dCas9) that binds to the *mrfp* DNA sequence to repress transcription. The mRFP expression cassette is located on the STAR expressing plasmid. (**b**) Fluorescence characterization (measured in units of fluorescence/optical density [OD] at 600 nm) was performed on *E. coli* cells transformed with a STAR variant 5 regulated sgRNA DNA plasmid in the absence (-STAR) or presence (+STAR) of a DNA plasmid encoding STAR variant 5. A DNA plasmid encoding a constitutively expressed sgRNA (+sgRNA) with no Csy4 hairpin was used as a positive control of repression. These conditions were performed in the absence (-dCas9) and the presence (+dCas9) of a DNA plasmid encoding the dCas9 protein. Data represents mean values of *n* = 9 biological replicas ± s.d.

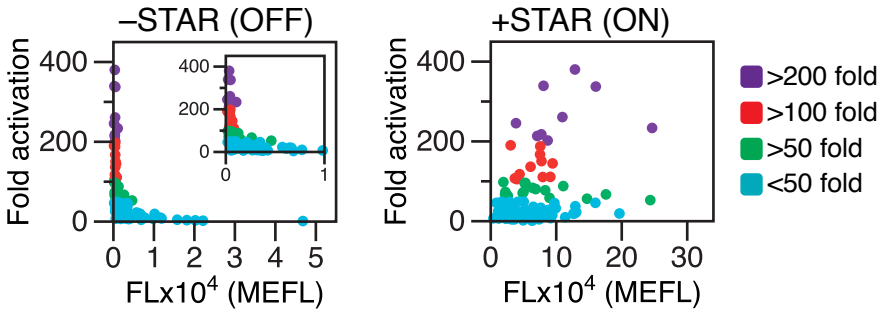


Supplementary Figure 21. Schematic of the STAR and CRISPRi genetic circuitry. (**a**) Schematic of STAR and CRISPRi NIMPLY (A AND NOT B) logic gate. Input A is a STAR that activates transcription of a target RNA regulated *mrfp* gene. Input B is a catalytically dead mutant of the Cas9 protein (dCas9) that in combination with a constitutively expressed single guide RNA (sgRNA) represses transcription of the *mrfp* gene. Only in the presence of input A and in the absence of input B will mRFP be expressed. (**b**) Schematic of DNA plasmids for the Incoherent Type 1 Feed Forward Loop (I1-FFL). (**c**) Schematic of temporal signal propagation through a STAR and CRISPRi I1-FFL[13] and (**d**) cartoon graphs of the resulting levels of I1-FFL species over time. AHL is added

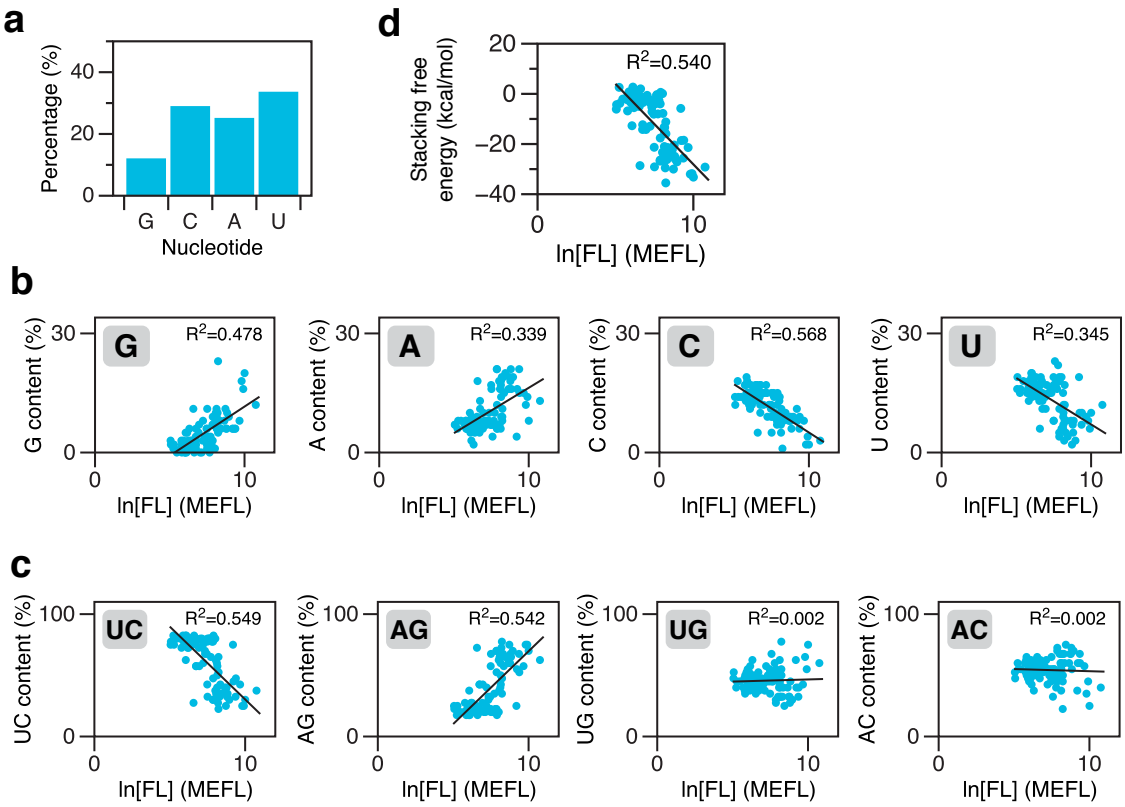
at t = 0 to induce STAR expression. STAR accumulates and at t = 1 an activation threshold is reached to activate mRFP transcription, resulting in an increase in mRFP levels. Simultaneously the sgRNA/dCas9 expression is activated and at t = 2 reaches a repression threshold that represses transcription of mRFP, resulting in decrease in mRFP levels. As a result the I1-FFL creates a pulse of mRFP expression[13]. In addition if mRFP is not completely repressed by CRISPRi, the I1-FFL should accelerate the response time towards steady-state compared to direct activation[13].



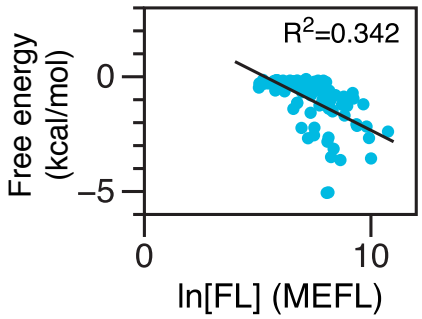
Supplementary Figure 22. Characterization of a STAR and CRISPRi incoherent type 1 feed-forward loop (I1-FFL). (**a**) Schematic of STARs and CRISPRi I1-FFL and direct activation circuits. (**b**-**d**) Fluorescence characterization (measured in units of fluorescence/optical density [OD] at 600 nm) was performed on *E. coli* cells transformed with plasmids encoding the I1-FFL or the direct activation (direct) cascade. At time 0 hour either acyl-homoserine lactone (+AHL) or water (-AHL) were added and fluorescence measured every 1 hour for 7 hours. Fluorescence data for each circuit were individually normalized by dividing by the final fluorescence values at 7 hours in the +AHL condition for each colony before calculating the mean and standard deviation at each time point. (**b**-**d**) Each panel represents n = 3 biological replicas ± s.d. collected on independent repeats for comparison.



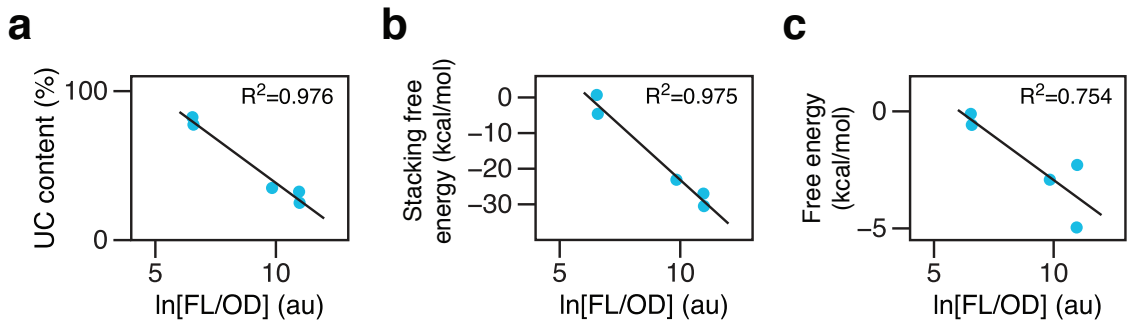
Supplementary Figure 23. STAR/target RNA characteristics governing dynamic range. Fluorescence characterization of each target RNA in the absence (–STAR) (left panel) and presence (+STAR) of cognate STAR (right panel) relative to fold of activation. Each target RNA variant is colored accordingly to the fold of activation. Fluorescence characterization data from **Figure 1b**. We note that STAR/target variant 101 was not considered because no activation was observed.



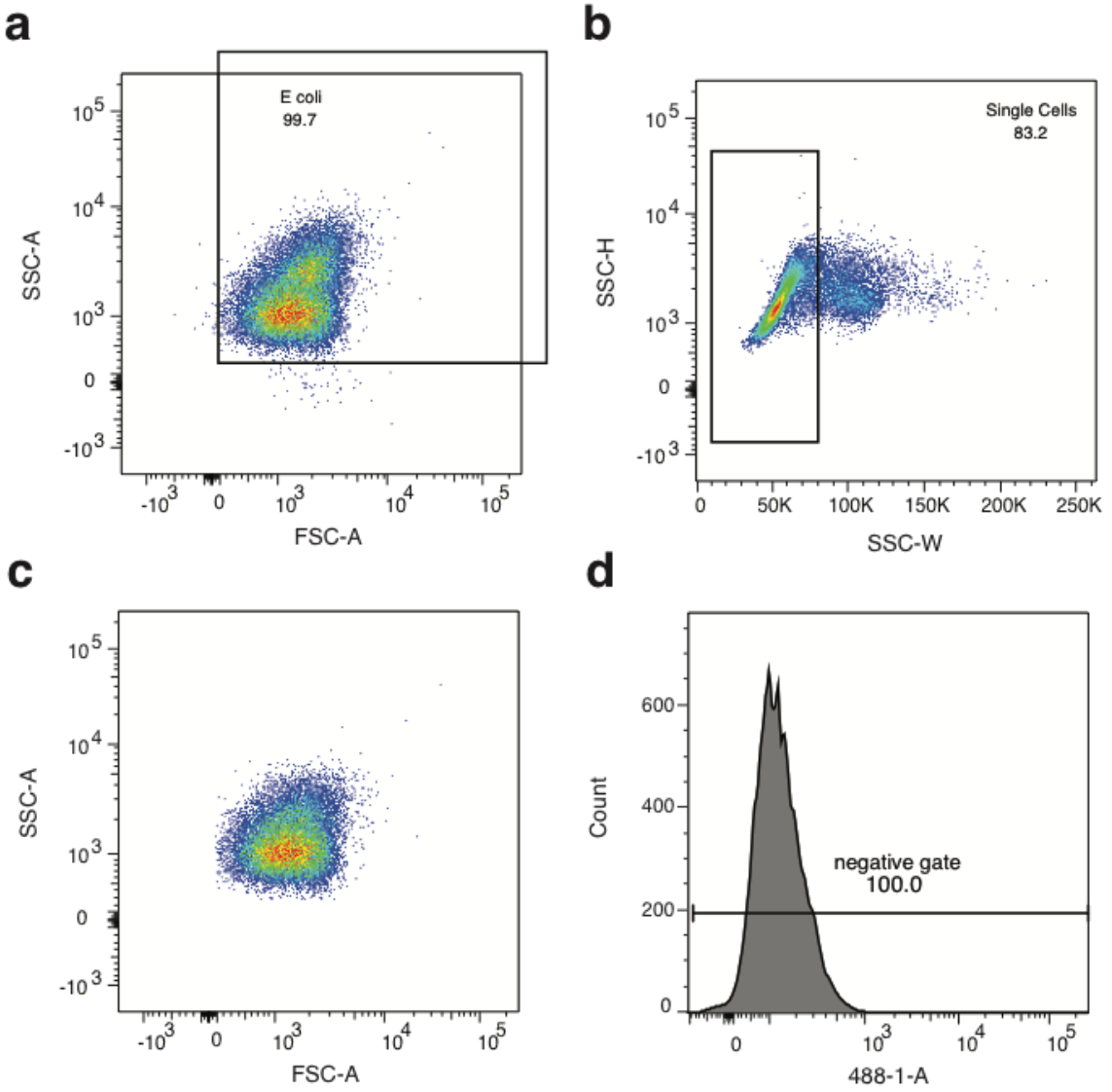
Supplementary Figure 24. Relationship between target RNA sequence composition/base stacking free energy and transcriptional termination efficiency. (**a**) Percentage nucleotide composition for the 100 computationally designed target RNA linear regions. (**b**) Relationship between the percentage content of single nucleotide (nucleotide identity shown in upper left of graph) or (**c**) double nucleotides (dinucleotide identity shown in upper left of graph) of the different target RNA linear regions and the natural log of fluorescence in the absence of STAR (OFF state). (**d**) Base stacking free energy of the different target RNA linear regions and the natural log of fluorescence in the absence of STAR (OFF state). Base stacking free energies were calculated using dinucleotide base stacking free energies previously determined[14]. Line of best fit is plotted and the correlation of determination (R^2) is displayed in upper right of graph. Natural log of fluorescence characterization data from **Fig. 1b**. We note that STAR/target variant 101 was not considered because no activation was observed.



Supplementary Figure 25. Relationship between predicted target RNA secondary structure and transcriptional termination efficiency. Secondary structure ensemble free energy of the different target RNA linear regions was predicted using NUPACK[12] and plotted against the natural log of fluorescence in the absence of STAR (OFF state). Line of best fit is plotted and the correlation of determination (R^2) is displayed in upper right of graph. Natural log of fluorescence characterization data from **Fig. 1b**. We note that STAR/target RNA variant 101 was not considered because no activation was observed.



Supplementary Figure 26. Relationship between transcriptional termination efficiency and sequence composition, base stacking free energy and predicted secondary structure of the ribA target RNAs. (**a**) Percentage content of uracil (U) and cytosine (C) nucleotides in the different target RNA linear regions and the natural log of fluorescence in the absence of STAR (OFF state). (**b**) Base stacking free energy of the different target RNA linear regions and the natural log of fluorescence in the absence of STAR (OFF state). Base stacking free energies were calculated using dinucleotide base stacking free energies previously determined[14]. (**c**) Ensemble free energy of the different target RNA linear regions was predicted using NUPACK[12] and plotted against the natural log of fluorescence in the absence of STAR (OFF state). Natural log of fluorescence characterization data from **Supplementary Fig. 7**.



Supplementary Figure 27. Flow cytometry gating. (**a**) *E. coli* gate based upon side scatter (SSC-A) and forward scatter (FSC-A) for an *E. coli* culture. (**b**) Single cell gate based upon side scatter pulse height (SSC-H) and side scatter pulse width (SSC-W). (**c**) Resulting population from *E. coli* gate and single cell gates combined. (**d**) Negative gate based upon fluorescence. See **Supplementary Note 4** for detailed description of gating used.

Supplementary Notes

Supplementary Note 1. Computational design of STARs using the NUPACK design tool

To computationally design STAR variants, we harnessed the NUPACK RNA design algorithm[11], a software suite for both the design and analysis of nucleic acid structures. To do this we used the NUPACK design web-application[10], running the NUPACK design script below. In brief, the script designs RNA sequences for the linear binding sequence and linear region of the STAR and target RNA, respectively, according to the design schematic in **Supplementary Fig. 2**. In this case, NUPACK designs STAR and target RNAs that are unstructured on their own but form a strong heteroduplex when present together. Certain sequences are prevented in designs and defects within the structure are allowed.

Designing

```
# STAR computational design script for NUPACK web-application

material = rna
temperature = 37.0
trials = 3
sodium[M] = 1.0
dangles = some
allowmismatch = true

structure STAR = ..........................................................

structure Target =
...............................................................................

structure ON =
((((((((((((((((((((((((((((((((((((((((((((((((((((((((+))))))))))))))))))))))))))))))))))))))
))))))))))))))))))))))))))))))))))))))).....................

domain a = N40

domain b = GCGGGGAATGTATACAGTTCATGTATATATTCCCCGCTTTTTTTTT

domain c = TGAACTGTATACATTCCCCGC

STAR.seq = c a*

Target.seq = a b

ON.seq = c a* a b

prevent = AAAA, CCCC, GGGG, UUUU, KKKKKK, MMMMMM, RRRRRR, SSSSSS, WWWWWW, YYYYYY

STAR.stop = 30.0
```

Supplementary Note 2. Determining STAR design principles

Our goal was to determine whether we could uncover design principles from the computationally designed STAR library. We began by understanding the relationship between target RNA regulatory characteristics and dynamic range. We first compared the fluorescence characterization of our target RNA library in both the absence (-STAR [OFF state]) and presence (+STAR [ON state]) of STAR, to the fold activation of each STAR/target variant (**Supplementary Fig. 23**). We note that variant 101 was excluded because this design gave rise to no significant activation (**Figure 1b**). While we saw a greater variation in the ON state fluorescence for designs with high-dynamic range, we observed that designs with high-dynamic ranges consistently had a low OFF level of fluorescence. In other words, target RNA transcriptional termination efficiency in absence of STAR appeared to be a key determinant of dynamic-range.

Given this, we next aimed to determine the relationship between target RNA sequence and structure, and the OFF state fluorescence, which we used as a proxy for transcriptional termination efficiency. We began by studying the effect of sequence composition of the computationally designed linear region of the target RNA (**Supplementary Fig. 24**). Overall we observed a relatively even distribution of the four nucleotides across the computationally designed target RNAs, with a slight decreased preference for guanosine (**Supplementary Fig. 24a**). We next compared how percentage content of each nucleotide affected OFF state fluorescence. We note that as percentage nucleotide content is often used as a proxy for free energy, we compared this to the natural log of fluorescence according to a model whereby the observed fluorescence is proportional to the equilibrium constant between the folded and un-folded states of the linear region (**Supplementary Fig. 24b**). This model effectively assumes equilibration of the linear region before termination, which could be valid given the fast timescales of RNA folding and the timescale of pausing of polymerase on a polyU tract. Interestingly we observed a relatively strong negative correlation (R^2 of ~0.5) between both uracil (U) and cytosine (C) percentage content and the OFF state fluorescence. Moreover, we observed a similar correlation when both U and C content were combined to give an R^2 of ~0.5 (**Supplementary Fig. 24c**). Taken together, this suggests that high UC content improves transcription termination efficiency of the target RNA. We hypothesized that this bias towards UC pyrimidine nucleotides for high termination efficiency may be attributed to base stacking interactions. Stacking interactions between the aromatic nucleotide bases are a major contributor to RNA structures. For example, in single-stranded RNAs base stacking has been shown to influence structural properties such as rigidity and formation of partial helical conformations[16]. Moreover, it is well-established that sequence is a major determinant of base stacking interactions, with decreasing stacking free energies in the order purine-purine, purine-pyrimidine, pyrimidine-purine and pyrimidine-pyrimidine[14]. Indeed, when we calculate the stacking free energy of different target RNA's linear regions according to previously determined stacking free energy values[14], we observed a

negative correlation to transcriptional termination efficiency (**Supplementary Fig. 24d**).

We next turned to the relationship between secondary structure and termination efficiency. Using NUPACK to predict secondary structure within the target RNA's linear region we compared the predicted ensemble free energy of each target RNA to the OFF state fluorescence (**Supplementary Fig. 25**). We observed a negative correlation with an R^2 of 0.342, suggesting that secondary structure within the linear region of the target RNA negatively impacts the transcription termination efficiency.

Taken together this suggested that both the presence of base stacking and secondary structure within the target RNA's linear region decreased transcription termination efficiency. We next sought to determine whether these observations represented a general STAR design principle or were specific for the AD1 terminator hairpin that was used as a terminator scaffold for the STAR library. To test this, we used NUPACK to computationally design a small library of STARs using the terminator from the *E. coli ribA* gene as a terminator scaffold[9]. This library was constructed and functionally characterized (**Supplementary Fig. 7**). We again observed a strong correlation between the OFF state fluorescence and UC content (R^2 0.976), stacking free energy (R^2 0.975) and ensemble free energy (R^2 0.754) of the target RNA's linear region and OFF state fluorescence (**Supplementary Fig. 26**). As such, this suggested that the negative impact of base stacking and secondary structures on transcription termination efficiency appeared to be a generalizable principle for the STAR regulatory system.

Supplementary Note 3. Predicting orthogonal STAR libraries

To predict orthogonal STAR/target RNA pairs, we developed an in house algorithm that uses NUPACK[15] to model STAR/target interactions and select pairs with minimal interactions between non-cognate pairs. This algorithm first creates two NUPACK input files for each of the 101 target RNAs: `<prefix>.in` and `<prefix>.list` whereby <prefix> is the target variant identifier. The `<prefix>.in` file specifies the number of strands (101 STARs and 1 target RNA), the 101 STAR variant sequences and the specific target variant sequence, as shown below:

```
<prefix>.in file structure:
102 # number of strands
NNNNNNN # STAR variant 1 sequence
NNNNNNN # STAR variant 2 sequence
…
NNNNNNN # Target variant 1 sequence
1 # option not used
```

It should be noted that only the linear binding sequences and linear region of the STAR/target were used.

The `<prefix>.list` file specifies the strand composition of the complexes to be analyzed – in this case all the STAR variants against a single target RNA.

```
<prefix>.list file structure:
1 102 # Complex of sequence 1 and sequence 102 in <prefix>.in
(STAR variant 1 and target variant 1)
2 102 # Complex of sequence 2 and sequence 102 in <prefix>.in
(STAR variant 2 and target variant 1)
….
101 102 # Complex of sequence 101 and sequence 102 in <prefix>.in
(STAR variant 101 and target variant 1)
```

The partition function, equilibrium base-pairing probabilities and minimum free energy (MFE) structures of the STAR-target complexes are then calculated by running NUPACK locally using the test tube analysis complex function[11, 12] with the following options:

```
complexes -T 37 -material rna -pairs -mfe –degenerate <prefix>
```

This results in an output file called `<prefix.ocx-mfe>` `which` contained the predicted minimum free energy folds and a dot-bracket structure for each STAR-target complex folded. The algorithm then compiles the dot-bracket structures of each STAR-target complex and counts the nucleotides that are unpaired or involved in intramolecular structures within the STAR strand. This results in a count of unpaired nucleotides for all possible 10,201 complexes, from which the number of linear region predicted base pairing interactions between STAR and target are determined (**Supplementary Fig. 7**). Based upon experimental

characterization (**Supplementary Fig. 9**) we predicted that sets of STAR/target RNAs that showed less than 13 base pairs of interaction in the target RNA for one of the combinations (i.e. either STAR 1/target 2 or STAR 2/ target 1) would be orthogonal. To identify predicted orthogonal sets, we first sorted the list of pair interactions to identify pairs that were predicted to have less than 13 bases of interaction. Additional STAR/target combinations were added to each pair to identify combinations of three STAR/targets predicted to be orthogonal. This was repeated to identify sets of four, five, etc. pairs of orthogonal STAR/targets. An example is shown in **Supplementary Fig. 10** that shows a set of 6 STARs that were identified using this approach, and then experimentally validated (**Figure 1d**).

Supplementary Note 4. Flow cytometry gating

Flow cytometry data collection was performed as explained in the online methods. FlowJo (v10.2) software was used for analysis of flow cytometry data. The three gates shown in **Supplementary Fig. 27** were used for all samples. The first gate (*E. coli* gate) is based upon side scatter (SSC-A) and forward scatter (FSC-A) to gate for *E. coli* cells and to remove readings from small debris (**Supplementary Fig. 27a**). The second gate (single cells gate) was used to gate away multicellular aggregations so that only single cells remain (**Supplementary Fig. 27b**). This gate was based upon side scatter pulse height (SSC-H) and side scatter pulse width (SSC-W) and the resulting population shown in **Supplementary Fig. 27c**. The final gate (negative gate) based upon fluorescence was within the second gate and was used to remove any high negative values that can arise from fluorescence baseline subtraction error during data acquisition (**Supplementary Fig. 27d**). We note that the following variants 40, 41, 80, 89, 91 showed some level of bimodality in either the absence or presence of STAR. All other variants were unimodal.

References

1. Pedelacq, J.D., Cabantous, S., Tran, T., Terwilliger, T.C. & Waldo, G.S. Engineering and characterization of a superfolder green fluorescent protein. *Nature biotechnology* **24**, 79-88 (2006).
2. Andersen, J.B. et al. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Applied and environmental microbiology* **64**, 2240-2246 (1998).
3. Qi, L., Haurwitz, R.E., Shao, W., Doudna, J.A. & Arkin, A.P. RNA processing enables predictable programming of gene expression. *Nat Biotechnol* **30**, 1002-1006 (2012).
4. Qi, L.S. et al. Repurposing CRISPR as an RNA-Guided Platform for Sequence-Specific Control of Gene Expression. *Cell* **152**, 1173-1183 (2013).
5. Mutalik, V.K. et al. Precise and reliable gene expression via standard transcription and translation initiation elements. *Nature methods* **10**, 354-360 (2013).
6. Carrier, T.A. & Keasling, J.D. Library of synthetic 5' secondary structures to manipulate mRNA stability in Escherichia coli. *Biotechnol Prog* **15**, 58-64 (1999).
7. St-Pierre, F. et al. One-step cloning and chromosomal integration of DNA. *Acs Synth Biol* **2**, 537-541 (2013).
8. Green, A.A., Silver, P.A., Collins, J.J. & Yin, P. Toehold switches: de-novo-designed regulators of gene expression. *Cell* **159**, 925-939 (2014).
9. Chappell, J., Takahashi, M.K. & Lucks, J.B. Creating small transcription activating RNAs. *Nature chemical biology* **11**, 214-220 (2015).
10. Zadeh, J.N. et al. NUPACK: Analysis and design of nucleic acid systems. *Journal of computational chemistry* **32**, 170-173 (2011).
11. Wolfe, B.R., Porubsky, N.J., Zadeh, J.N., Dirks, R.M. & Pierce, N.A. Constrained Multistate Sequence Design for Nucleic Acid Reaction Pathway Engineering. *J Am Chem Soc* **139**, 3134-3144 (2017).
12. Dirks, R.M., Bois, J.S., Schaeffer, J.M., Winfree, E. & Pierce, N.A. Thermodynamic analysis of interacting nucleic acid strands. *Siam Rev* **49**, 65-88 (2007).
13. Alon, U. Network motifs: theory and experimental approaches. *Nat Rev Genet* **8**, 450-461 (2007).
14. Jafilan, S., Klein, L., Hyun, C. & Florian, J. Intramolecular base stacking of dinucleoside monophosphate anions in aqueous solution. *J Phys Chem B* **116**, 3613-3618 (2012).
15. Zadeh, J.N., Wolfe, B.R. & Pierce, N.A. Nucleic acid sequence design via efficient ensemble defect optimization. *Journal of computational chemistry* **32**, 439-452 (2011).
16. Seol, Y., Skinner, G.M., Visscher, K., Buhot, A. & Halperin, A. Stretching of homopolymeric RNA reveals single-stranded helices and base-stacking. *Phys Rev Lett* **98**, 158103 (2007).