

Supplementary Materials

Supplementary Table S1. DNA sequences for the transcriptional regulation system.

Application	Name	Sequence
SWT Sensor	JS1	TCCATCTCGCTCGTTCCGTCTCCTTCTCTTTTCCTTTTCG CAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	JS2	CTGACCCGAGCAACCACCTATACCTTAAACCGACACT AGCAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	JS3	GCGGCGAACTCCCAACACAGCAACCTACTCACAACCT CTTAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	JS4	CGCCGGGCTACCTCACACCGGATTCTACTACAT TTCAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	JS5	GGAGTAAAGGGTAGAGTAAAGGTGAGGGGAAGGGTG GTTGAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	JS6	GCAGTTTTTTAGTCTCTCGGCGCTTACGATGCGGCGTT TCAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	JS7	AGTAATCATTCAAGGCGGTTTCAGCGGGCGAGTAGCGT AGTAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	JS8	GGGGGAGGAAATGGAGAGGAGAAAGAGAAGGGAAA ATGGGAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	JS9	CCGTGTTAGTAAGTTGGGCCGTGTGAGGTTGTTGTGT TCGAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	JS10	CTCCTTTTCTCTCCCTCTCTCTCTCTTCCCCTCCCTTTC TAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	JS11	GCGCTGGCCGACAGAAAGGCCCCATAAAGACTGAAC ATTAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	JS12	CACACACACACACCACAGGCACAGACGACAAATACA CACCAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	ZS1	ACCACATTCTCATCACCCCCCTTCCTCACACTTTTCA CAAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	ZS15	GTCATCCGTCATCCATTTCGTCATCCATCGTTCGCTCAT CCAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	ZS16	ACACAATCAATCAACACCTCTACAAAACACTACACAA AAACGGCCCCCTCGGTAACGAGGGGCCGTTTTTTT
	ZS17	CGTCCGTCAAGCCATCATCCAGTCCATCCATCCAGTT CTAAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	ZS18	GACCTACCTGACCTACCTGCTGCTACTACGACGAAC TACAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	ZS19	CCACGTACTAACTCTCATCACTCACGCACTCATCGAC TATAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	ZS20	TCCTGAACCCTATATACAATAATTCTAACACGTCCTC GAAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	ZS21	GTCATCCGTCATCCATTTCGTCATCCATCGTTCGCTCAT CCAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT
	ZS22	GACCTACCTACTACTACTACCTACTGACCTACGTGCCT ACAAAGCCCCGCCGAAAGGCGGGCTTTTTTTT

SWT Trigger	JT1	GGCGGGCTTTGCGAAAGGAAAAGAGAAGGAGACGGA ACGAGCGAGATGGA
	JT2	GGCGGGCTTTGCTAGTGTCTGGTTTAAGGTATAGGTGG TTGCTCGGGTCAG
	JT3	GGCGGGCTTTAAGAAGTTGTGAGTAGGTTGCTGTGTT GGGAGTTCGCCGC
	JT4	GGCGGGCTTTGAAATGTAGTAGAATGAATCCGGTGTG AGGTAGCCCGGCG
	JT5	GGCGGGCTTTCAACCACCCTTCCCCTCACCTTTACTCT ACCCTTTACTCC
	JT6	GGCGGGCTTTGAAACGCCGCATCGTAAGCGCCGAGA GACTAAAAAACTGC
	JT7	GGCGGGCTTTACTACGCTACTCGCCCGCTGAACCGCC TTGAATGATTACT
	JT8	GGCGGGCTTTCCCATTTTCCCTTCTCTTTCTCCTCTCCA TTTCTCCCCC
	JT9	GGCGGGCTTTCGAACACAACAACCTCACACGGCCCAA CTTACTAACACGG
	JT10	GGCGGGCTTTAGAAAGGGAGGGGAAGAGAGAGAGA GGGAGAGAAAAGGAG
	JT11	GGCGGGCTTTAAATGTTTCAGTCTTTATGGGGCCTTTCT GTCGGCCAGCGC
	JT12	GGCGGGCTTTGGTGTGTATTGTCTGTGTGCCTGTGG TGTGTGTGTGTG
	ZT1	GGCGGGCTTTTGTGAAAAGTGTGAGGAAGGGGGGTG ATGAGGAATGTGGT
	ZT15	GGCGGGCTTTGGATGAGCGAACGATGGATGACGAAT GGATGACGGATGAC
	ZT16	CGAGGGGCCGTTTTTGTGTAGTGTTTTGTAGAGGTGTT GATTGATTGTGT
	ZT17	GGCGGGCTTTTAGAACTGGATGGATGGACTGGATGAT GGCTTGACGGACG
	ZT18	GGCGGGCTTTGTAAAGTTCGTCTAGTAGCAGCAGGTA GGTCAGGTAGGTC
	ZT19	GGCGGGCTTTATAGTCGATGAGTGCGTGAGTGATGAG AGTTAGTACGTGG
	ZT20	GGCGGGCTTTTCGAGGACGTGTTAGAATTAGTTGTAT ATAGGGTTCAGGA
	ZT21	GGCGGGCTTTGGATGAGCGAACGATGGATGACGAAT GGATGACGGATGAC
	ZT22	GGCGGGCTTTGTAGGCACGTAGGTCAGTAGGTTAGTAG TAGTAGGTAGGTC
6-layer cascade	ZS1-ZT16	ACCACATTCTCATCACCCCCCTTCCTCACACTTTTCA CAAAAGCCCGCCGAAAGGCGGGCTTTTTTTTCGATCG AGGGGCCGTTTTTGTGTAGTGTTTTGTAGAGGTGTTG ATTGATTGTGT

	ZS16-JT2	ACACAATCAATCAACACCTCTACAAAACACTACACAA AAACGGCCCCCTCGGTAACGAGGGGCCGTTTTTTTTTCG ATGGCGGGCTTTGCTAGTGTGCGTTTAAGGTATAGGT GGTTGCTCGGGTCAG
	JS2-ZT22-	CTGACCCGAGCAACCACCTATACCTTAAACCGACACT AGCAAAGCCCGCCGAAAGGCGGGCTTTTTTTTTTCGATG GCGGGCTTTGTAGGCACGTAGGTCAGTAGGTAGTAGT AGTAGGTAGGTC
	ZS22-ZT19	GACCTACCTACTACTACTACCTACTGACCTACGTGCCT ACAAAGCCCGCCGAAAGGCGGGCTTTTTTTTTTCGATGG CGGGCTTTATAGTCGATGAGTGCGTGAGTGATGAGAG TTAGTACGTGG
	ZS19-3WJdB	CCACGTACTAACTCTCATCACTCACGCACTCATCGAC TATAAAGCCCGCCGAAAGGCGGGCTTTTTTTTTTGACC CACATACTCTGATGATCCGAGACGGTCGGGTCCAGAT ATTCGTATCTGTGCGAGTAGAGTGTGGGCTCGGATCAT TCATGGCAAGAGACGGTCGGGTCCAGATATTCGTATC TGTCGAGTAGAGTGTGGGCTCTTGCCATGTGTATGTG GG
SWT AND gate	Sensor: ZS22-ZS19	GACCTACCTACTACTACTACCTACTGACCTACGTGCCT ACAAAGCCCGCCGAAAGGCGGGCTTTTTTTTTAGGGCC ACGTACTAACTCTCATCACTCACGCACTCATCGACTA TAAAGCCCGCCGAAAGGCGGGCTTTTTTTTT
	Trigger 1: ZT22	GGCGGGCTTTGTAGGCACGTAGGTCAGTAGGTAGTAG TAGTAGGTAGGTC
	Trigger 2: ZT19	GGCGGGCTTTATAGTCGATGAGTGCGTGAGTGATGAG AGTTAGTACGTGG
	Sensor 1: ZS1-JS2- AATS(ZS19)	ACCACATTCTCATCACCCCCCTTCCTCACACTTTTCA CAAAAGCCCGCCGAAAGGCGGGCTTTTTTTTTCTGACC CGAGCAACCACCTATACCTTAAACCGACACTAGCAAA GCCCGCCGAAAGGCGGGCTTTTTTTTTAGGGCCACGTA CTAACTCTCATCACTCACGCACTCATCGACTATAAAG CCCGCC
SWT NAND gate	Sensor 2: ATS(ZS19)- ZS19	GGCGGGCTTTATAGTCGATGAGTGCGTGAGTGATGAG AGTTAGTACGTGGCCACGTACTAACTCTCATCACTCA CGCACTCATCGACTATAAAGCCCGCCGAAAGGCGGG CTTTTTTTTT
	Trigger 1: ZT1	GGCGGGCTTTTGTGAAAAGTGTGAGGAAGGGGGGTG ATGAGGAATGTGGT
	Trigger 2: JT2	GGCGGGCTTTGCTAGTGTGCGTTTAAGGTATAGGTGG TTGCTCGGGTCAG
	Trigger: AATS(ZS19)	CCACGTACTAACTCTCATCACTCACGCACTCATCGAC TATAAAGCCCGCC
SWT NOT gate	Sensor: ATS(ZS19)- ZS19	GGCGGGCTTTATAGTCGATGAGTGCGTGAGTGATGAG AGTTAGTACGTGGCCACGTACTAACTCTCATCACTCA CGCACTCATCGACTATAAAGCCCGCCGAAAGGCGGG CTTTTTTTTT

SWT NOR gate	Sensor: ATS(ZS19)- ZS19- ATS(JS2)-JS2	GGCGGGCTTTATAGTCGATGAGTGCGTGAGTGATGAG AGTTAGTACGTGGCCACGTACTAACTCTCATCACTCA CGCACTCATCGACTATAAAGCCCGCCGAAAGGCGGG CTTTTTTTTCGATCCGCCCCGAAACGATCACAGCCAAA TTCCATATCCACCAACGAGCCCAGTCGACTGGGCTCG TTGGTGGATATGGAATTTGGCTGTGATCGTTTCGGGC GGCTTTCCGCCCCGAAAAAAAAA
	Trigger 1: AATS(ZS19)	CCACGTACTAACTCTCATCACTCACGCACTCATCGAC TATAAAGCCCGCC
	Trigger 2: AATS(JS2)	CTGACCCGAGCAACCACCTATACCTTAAACCGACACT AGCAAAGCCCGCC
	TBA15	GGTTGGTGTGGTTGG
Thrombin aptamer	TBA25	GGAACAAAGCTGAAGTACTTACCC
	TBA29	AGTCCGTGGTAGGGCAGGTGGGGGTGACT
	TBA61	TACAGGCAGTAAGGCCGTGTCATCAGGATGGGCACTT TCCTAGGGCAGGTTAGCAACAGGA
	TBA25-ZS1	GGAACAAAGCTGAAGTACTTACCCACCACATTCCTC ATCACCCCCCTTCCTCACACTTTTCACAAAAGCCCGCC GAAAGGCGGGCTTTTTTTT
APT-SWT AND gate	TBA25- Hairpin- Broccoli-ZS1	GGAACAAAGCTGAAGTACTTACCCGCATACAGTCCA TCATTCAGGCAGGGATAACTGAATGATGGACCCGTCC TACTAGATAGCGACGCGACCGAGACGGTCGGGTCCA GATATTCGTATCTGTTCGAGTAGAGTGTGGGCTCGGTC GCGTCTAGCACACATTTCCTCATCACCCCCCTTCCTCA CACTTTTCACAAAAGCCCGCCGAAAGGCGGGCTTTTT TTT
	Sensor 1: TBA25-ZT19	GGAACAAAGCTGAAGTACTTACCCGCTAGGCGGGC TTTATAGTCGATGAGTGCGTGAGTGATGAGAGTTAGT ACGTGG
	Sensor 2: ZS19-3WJdB	CCACGTACTAACTCTCATCACTCACGCACTCATCGAC TATAAAGCCCGCCGAAAGGCGGGCTTTTTTTTGGACC CACATACTCTGATGATCCGAGACGGTCGGGTCCAGAT ATTCGTATCTGTTCGAGTAGAGTGTGGGCTCGGATCAT TCATGGCAAGAGACGGTCGGGTCCAGATATTCGTATC TGTCGAGTAGAGTGTGGGCTCTTGCCATGTGTATGTG GG
	Broccoli	GACGCGACCGAGACGGTCGGGTCCAGATATTCGTATC TGTCGAGTAGAGTGTGGGCTCGGTTCGCGTC
Reporter gene	3WJdB	GGACCCACATACTCTGATGATCCGAGACGGTCGGGTC CAGATATTCGTATCTGTTCGAGTAGAGTGTGGGCTCGG ATCATTTCATGGCAAGAGACGGTCGGGTCCAGATATTC GTATCTGTTCGAGTAGAGTGTGGGCTCTTGCCATGTGT ATGTGGG

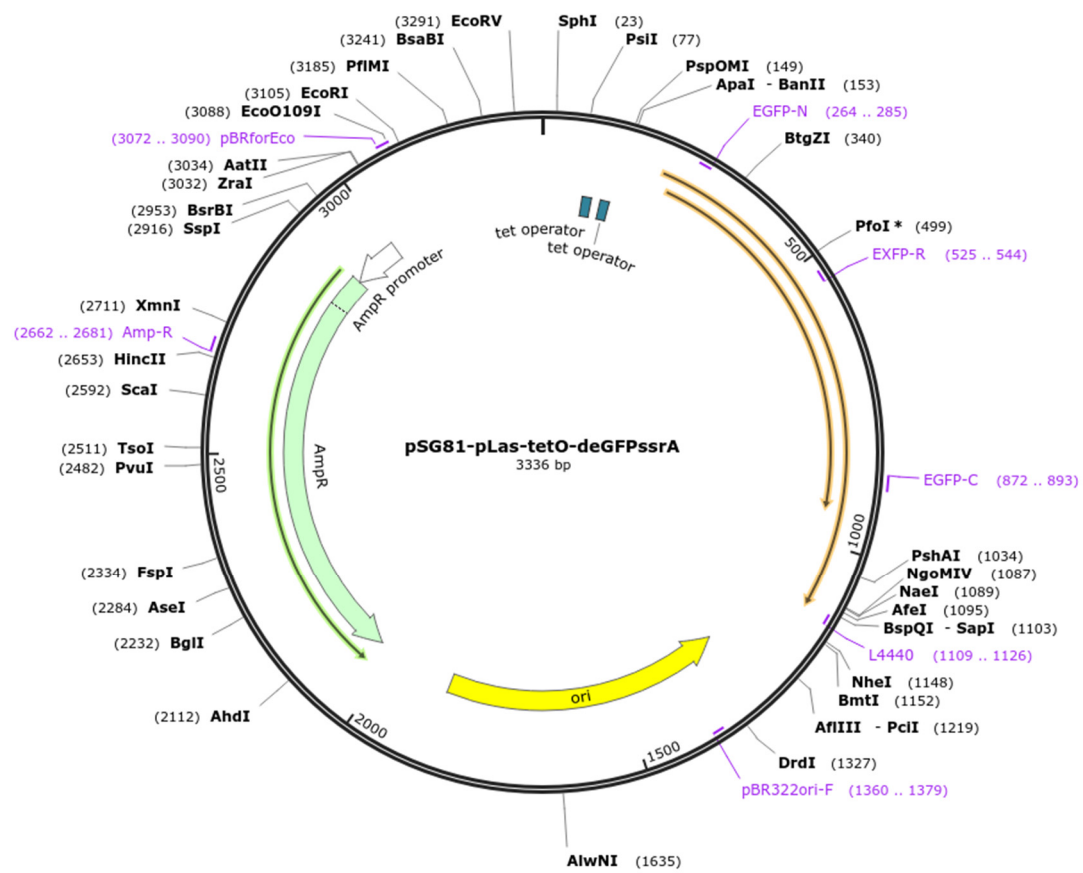


Figure S1. Plasmid map of pSG81. Expresses deGFP fluorescent protein with ssrA degradation tag in *E. coli*. The expression is controlled by a pLas-tetO combinatorial promoter. This plasmid was used in this study for the construction of recombinant plasmids involved in *in vitro* transcription.

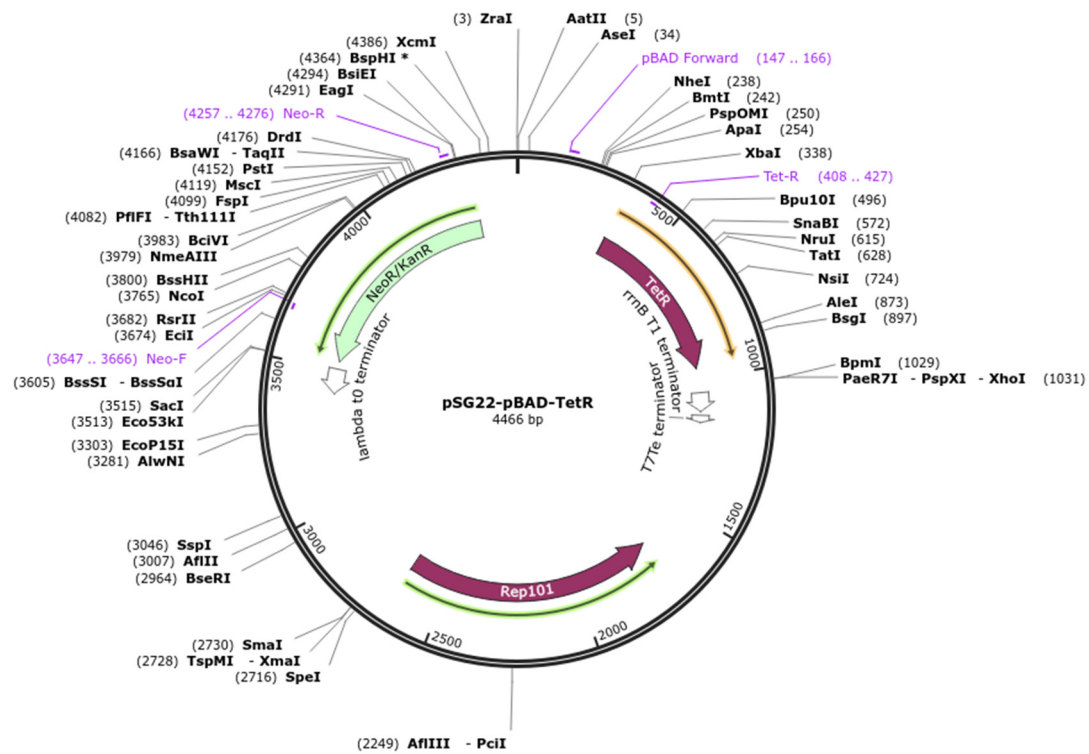
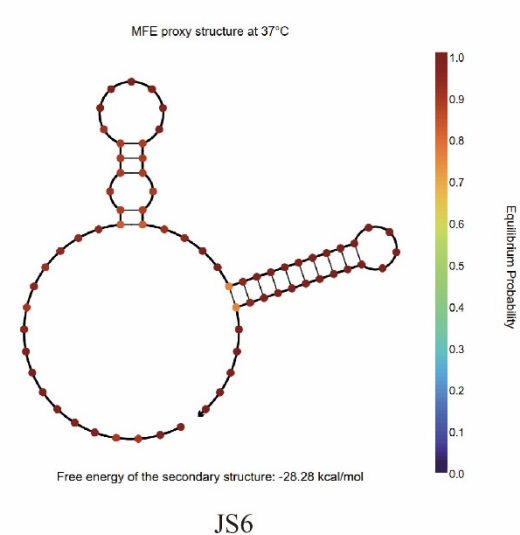
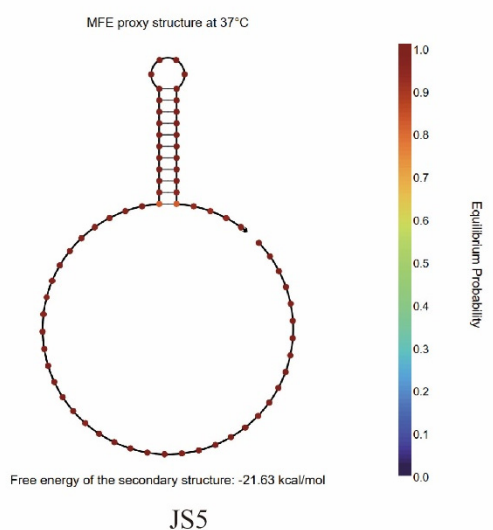
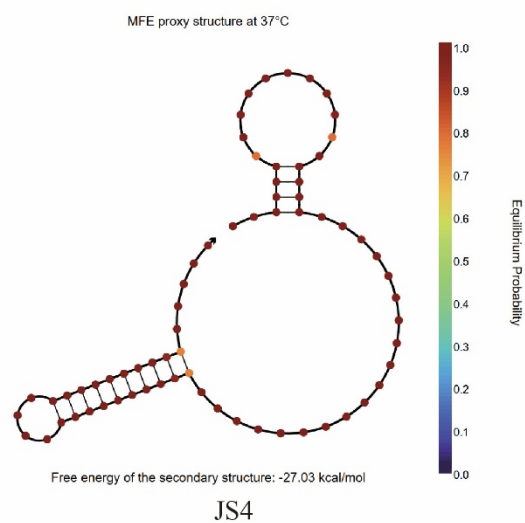
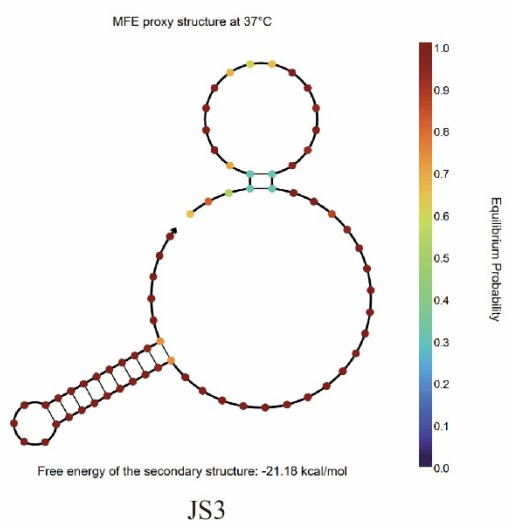
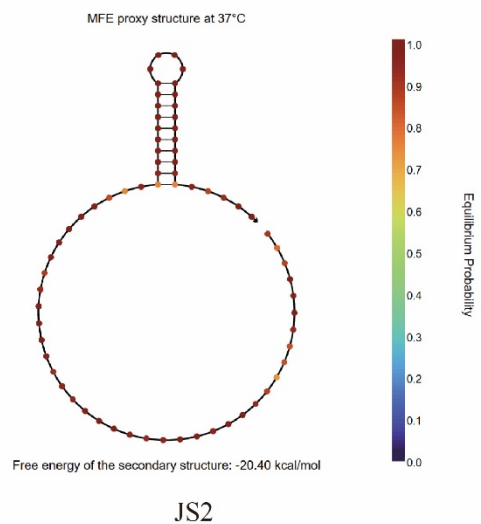
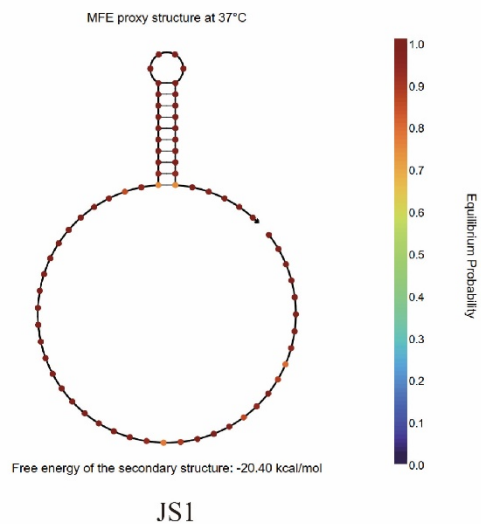
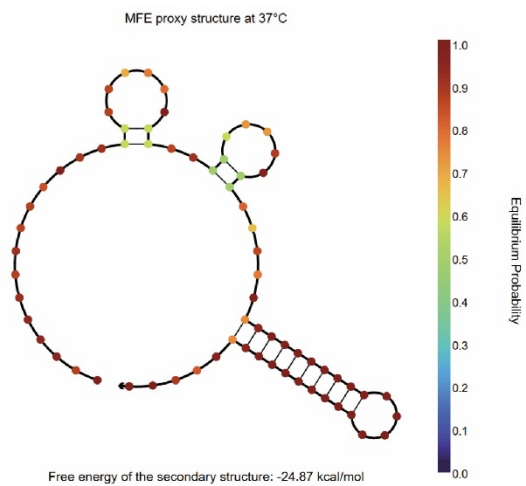
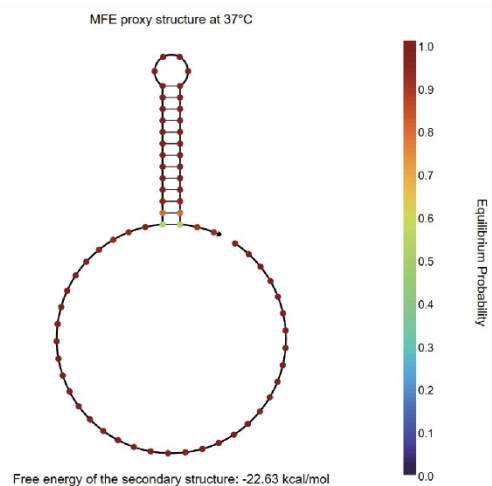


Figure S2. Plasmid map of pSG22. Expresses TetR protein in *E. coli*. The expression is controlled by a pBAD promoter. This plasmid was used to verify the transcriptional regulation performance of SWT in *E. coli*.

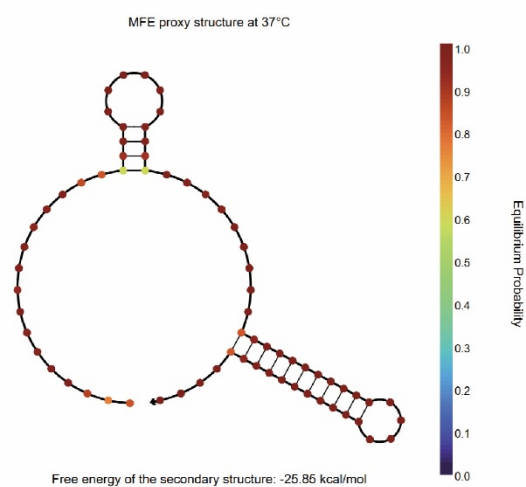




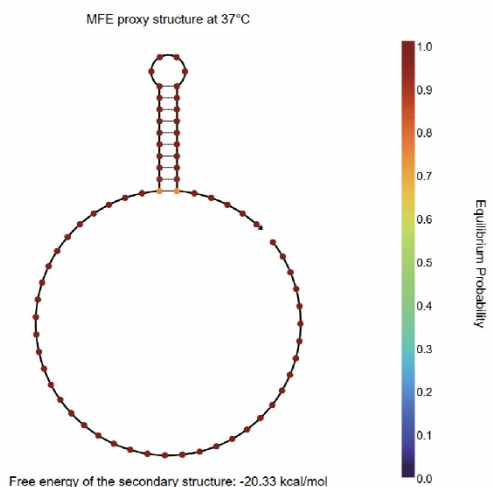
JS7



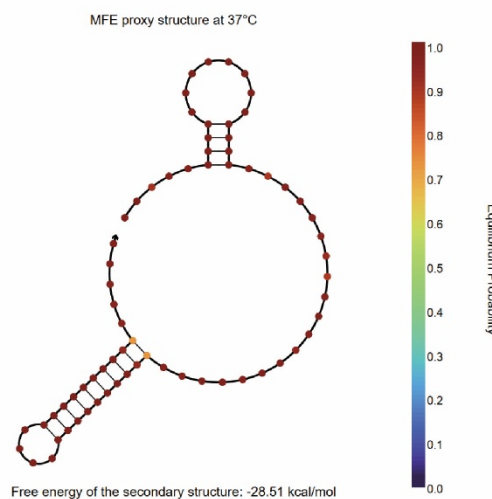
JS8



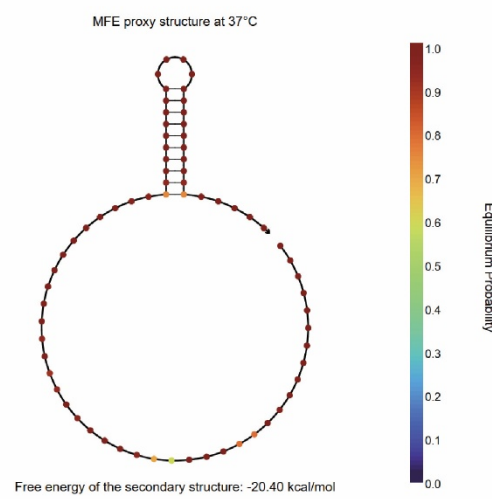
JS9



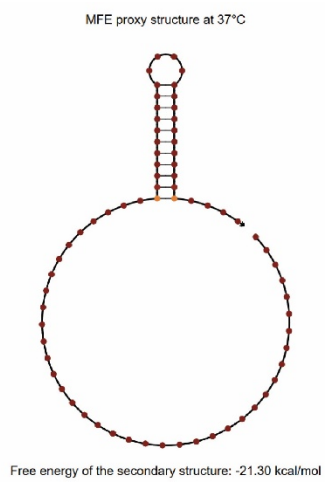
JS10



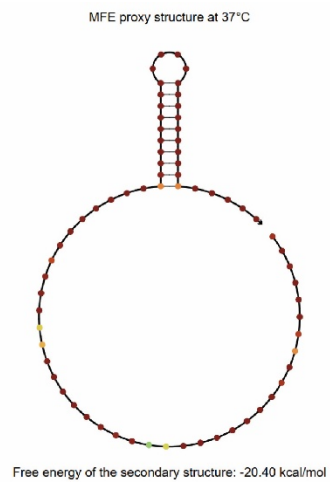
JS11



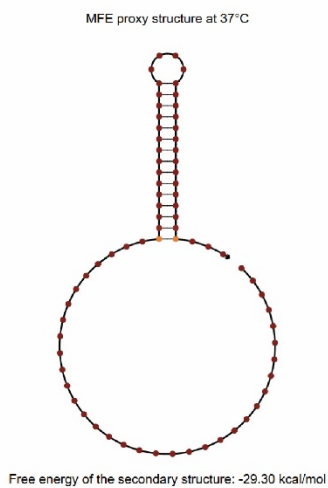
JS12



ZS1



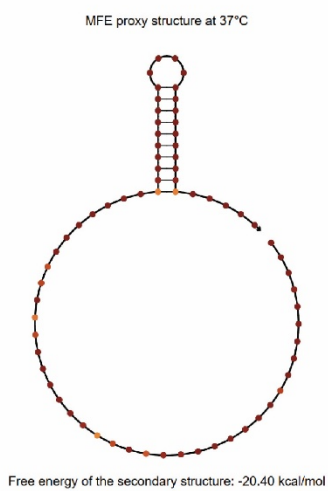
ZS15



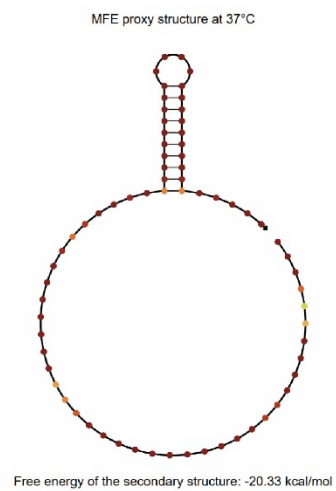
ZS16



ZS17



ZS18



ZS19

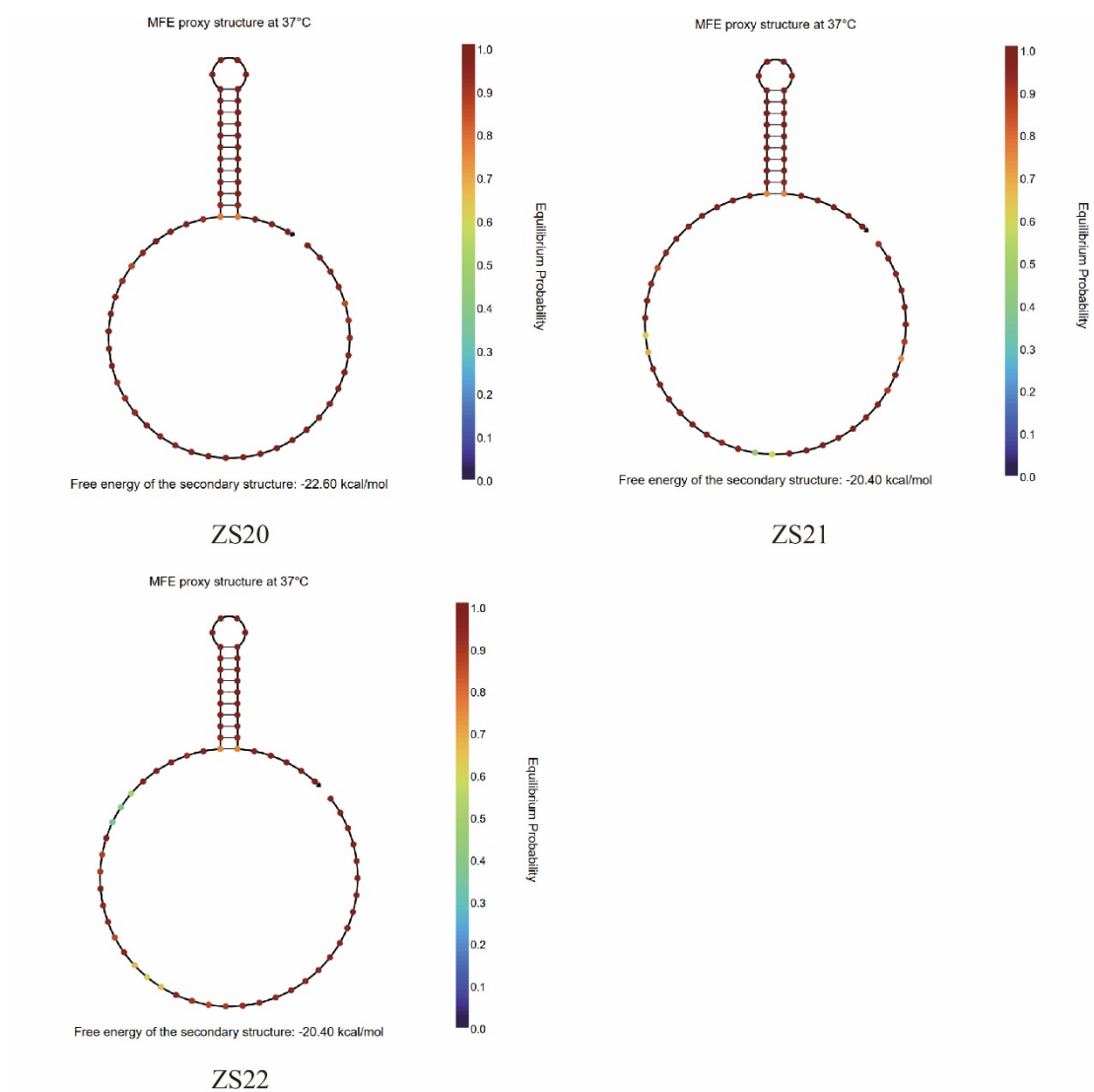


Figure S3. Secondary structure predictions of 21 SWT variants generated by the algorithm. The secondary structures of the 21 SWT variants generated by the algorithm were predicted, and all except JS3, JS4, JS6, JS7, JS9, and JS11 matched the theoretical structures.

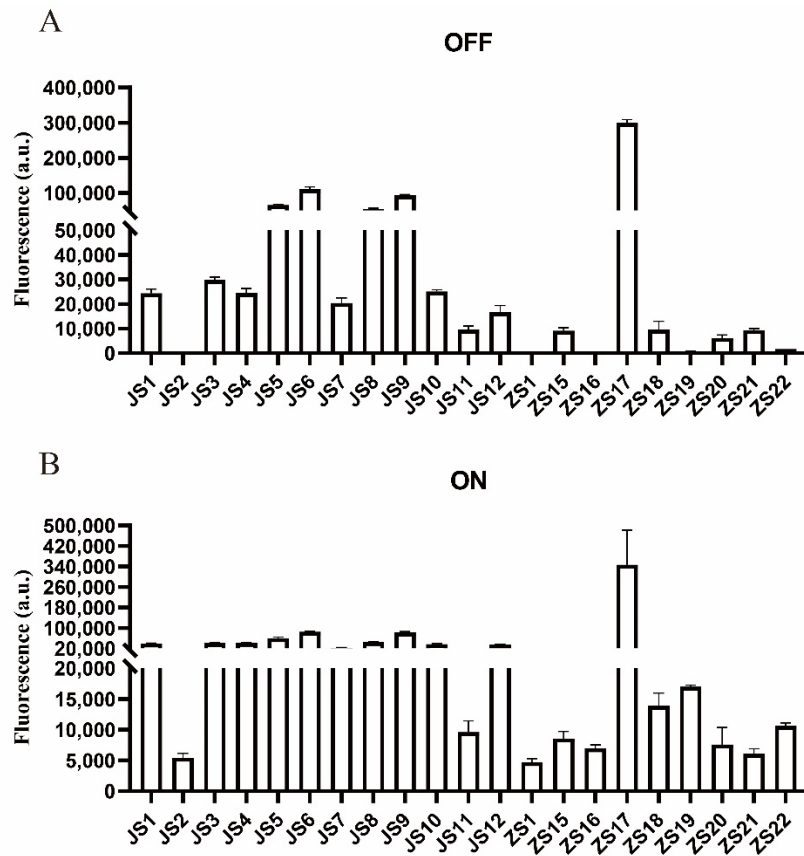


Figure S4. Fluorescence values of SWT under different states. The final concentrations of the linear sensor and trigger RNA templates are both 10 nM. (A) The fluorescence value in the OFF state reflects the leakage expression of SWT itself. The lower the leakage value, the stronger the transcriptional repression ability of SWT. (B) The fluorescence value in the ON state is the activation of transcription after introducing the trigger RNA. The lower the leakage fluorescence in the OFF state and the higher the activation fluorescence in the ON state, the better the performance of the SWT. Error bars indicate the average value of three independent biological replicates $\pm$ s.d.

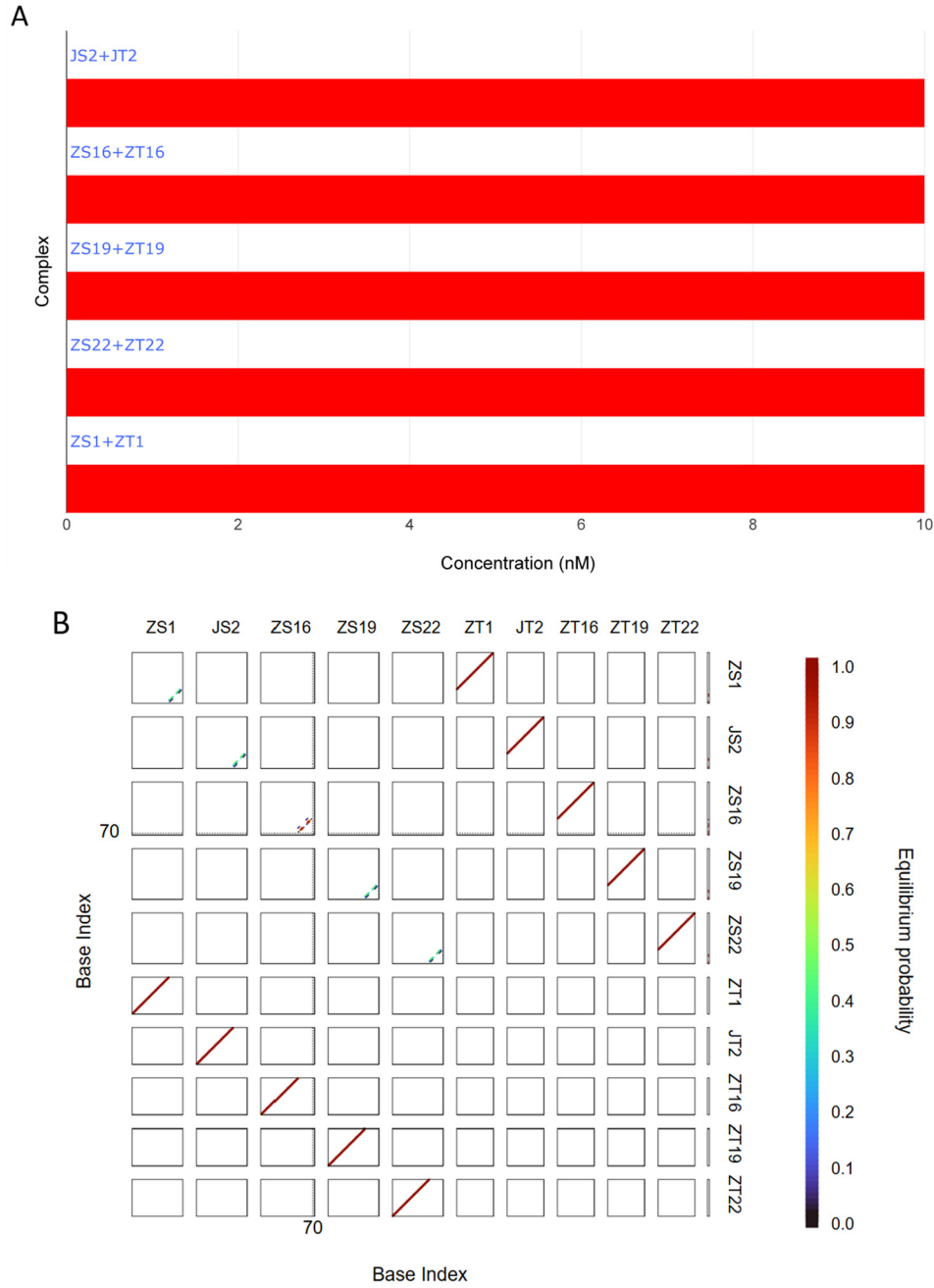


Figure S5. Orthogonality prediction of candidate SWT. The orthogonal binding of 5 pairs of candidate SWTs (trigger and sensor RNA concentrations both set to 10 nM) in the same test tube collection was predicted using the NUPACK online software. **(A)** The equilibrium concentration of complexes reflects the equilibrium concentration of each complex in the test tube collection at a given total initial concentration. **(B)** Reflects the pairing probability between different bases in the test tube collection, where the area and color of the point at the *i*-th row and *j*-th column are proportional to the equilibrium score of base *i* pairing with base *j* in the test tube solution (scores below 0.001 are not displayed). The darker the color, the higher the pairing probability.

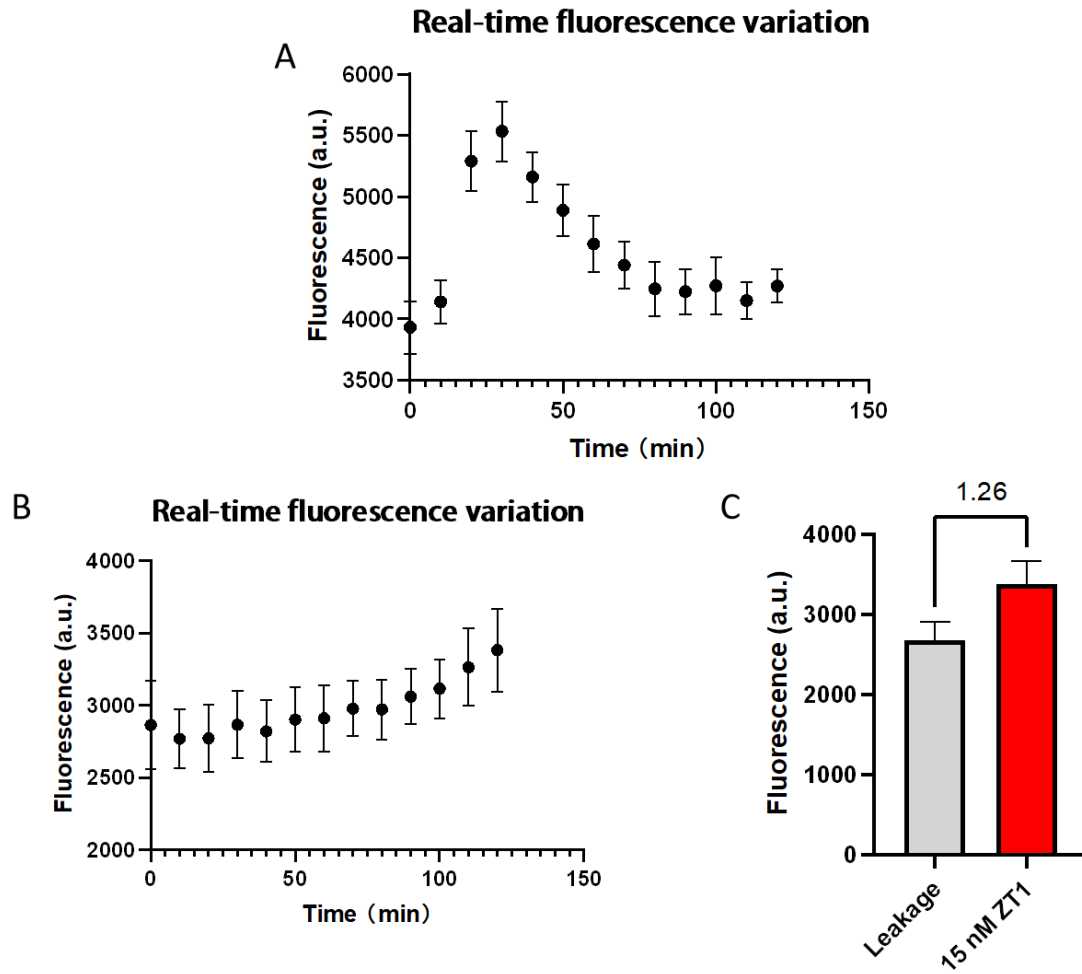


Figure S6. Six-layer cascade characterization. (A) Shows the real-time fluorescence change when all templates are transcribed together in the same system using the One-pot method, with the fluorescence peak reached around 30 minutes, indicating rapid reaction. (B-C) Shows the real-time fluorescence change and fluorescence peak comparison under the Two-pots method when the concentration of signal input module X is 15 nM, the concentration of signal conversion module Y is 5 nM, and the concentration of signal output module Z is 5 nM. Error bars indicate the average value of three independent biological replicates $\pm$ s.d.

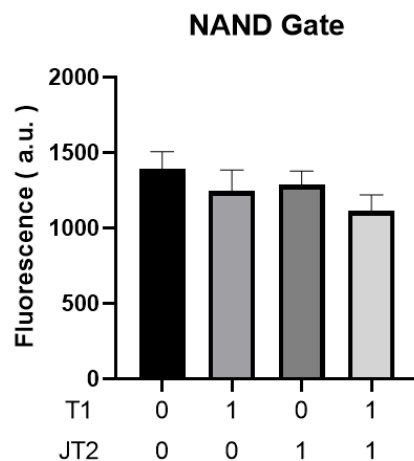
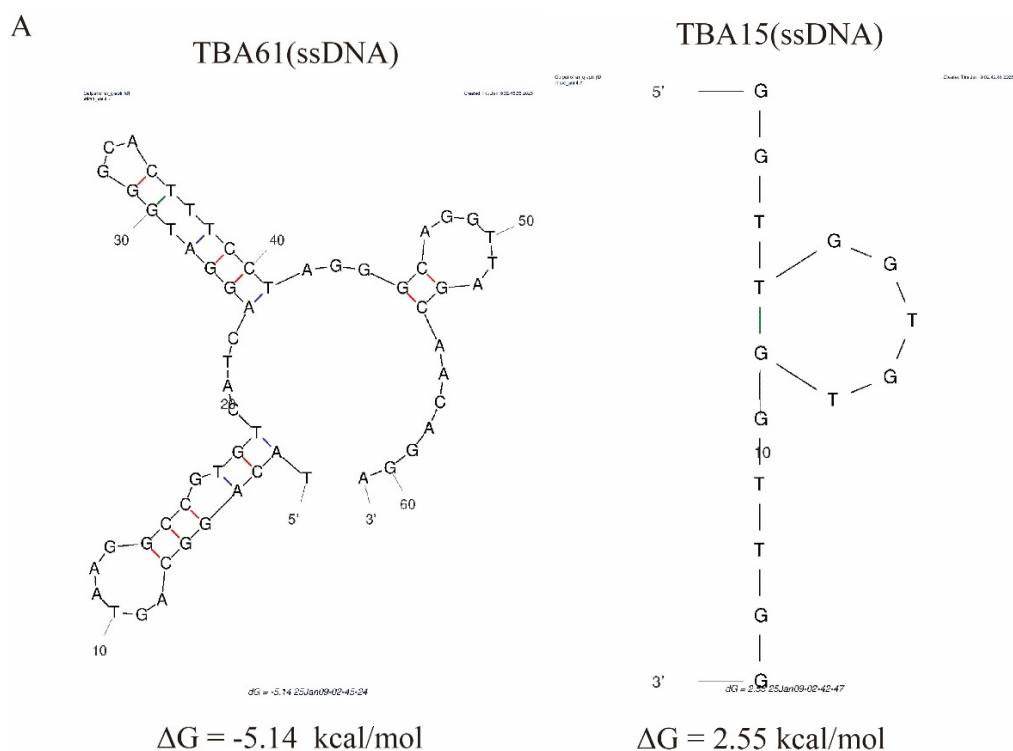
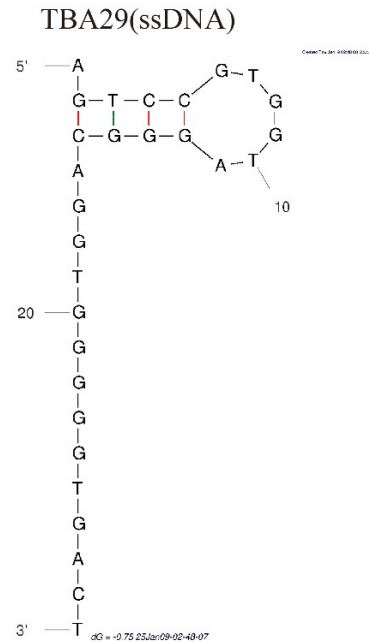
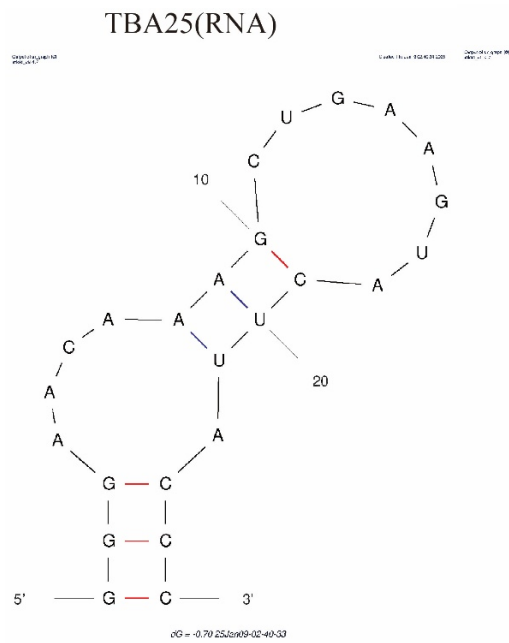


Figure S7. Characterization of the NAND gate using one-pot method. NAND Gate (One-pot) characterization shows the transcription of all NAND Gate templates in the same *in vitro* transcription system (where the input module template DNA concentration is 20 nM, and the signal conversion module and output module template DNA concentrations are both 10 nM). Error bars indicate the average value of three independent biological replicates $\pm$ s.d.

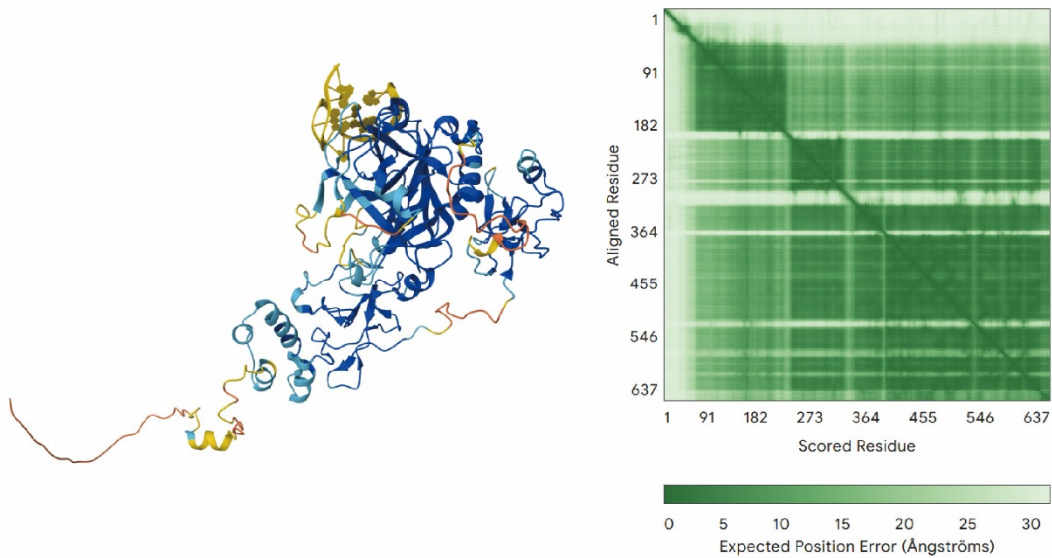




B

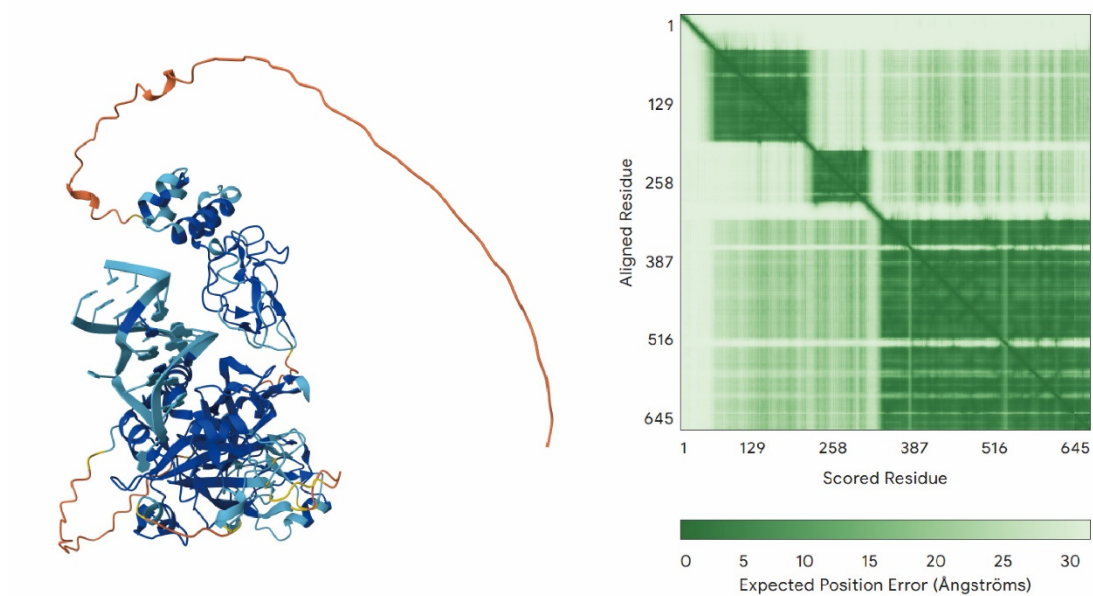


TBA15-Ligand



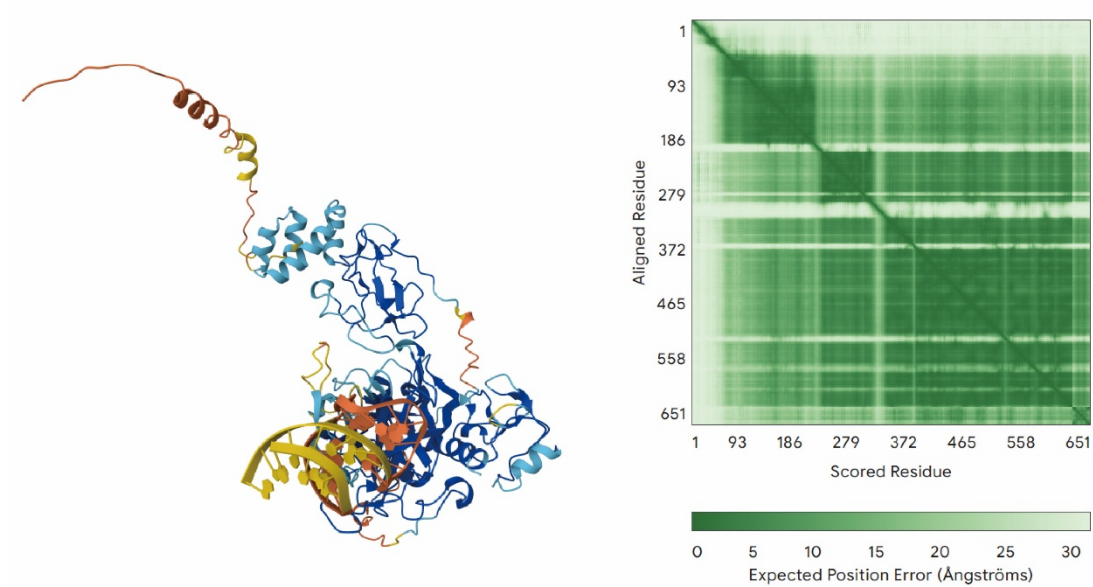
ipTM = 0.85 pTM = 0.76

TBA25-Ligand



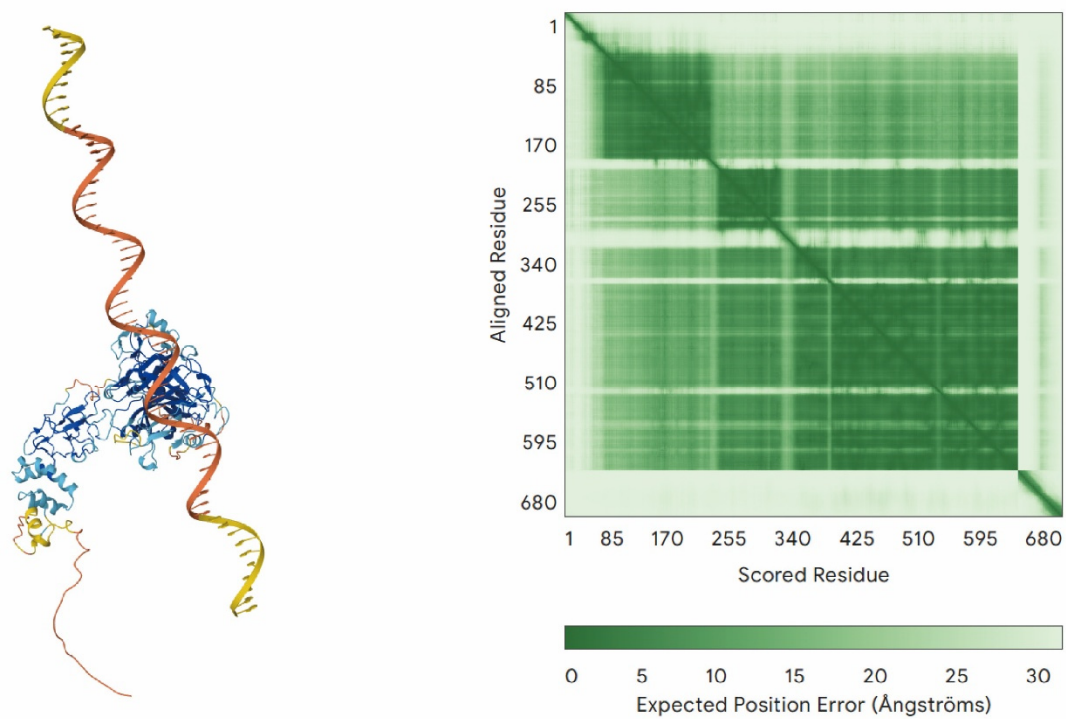
ipTM = 0.93 pTM = 0.58

TBA29-Ligand



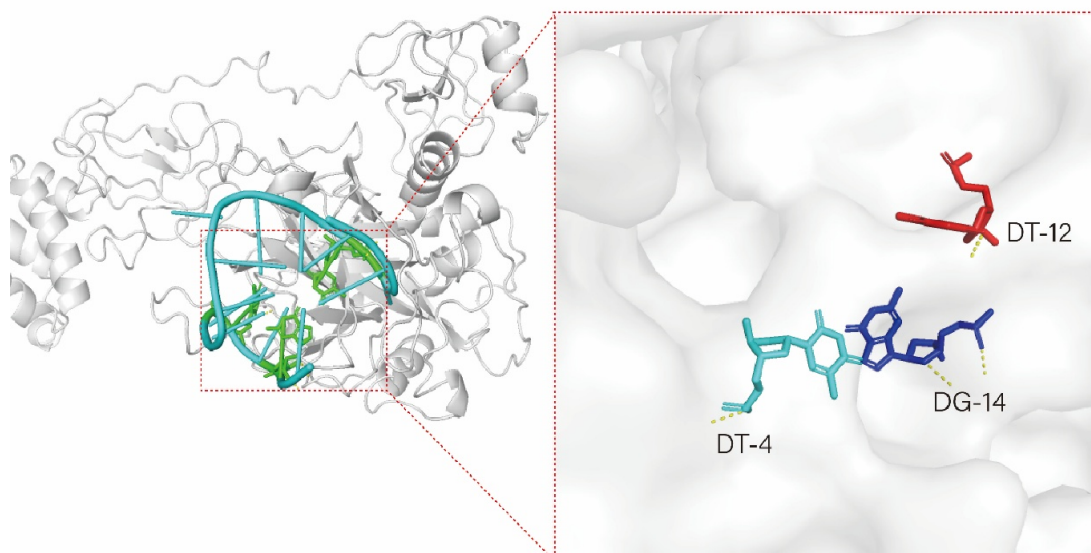
ipTM = 0.7 pTM = 0.74

TBA61-Ligand

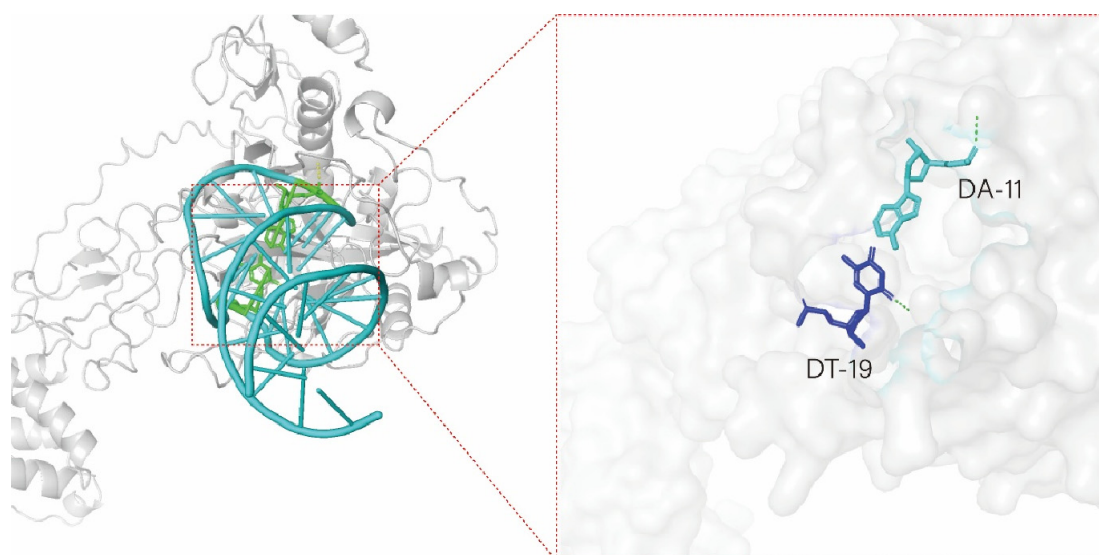


ipTM = 0.11 pTM = 0.69

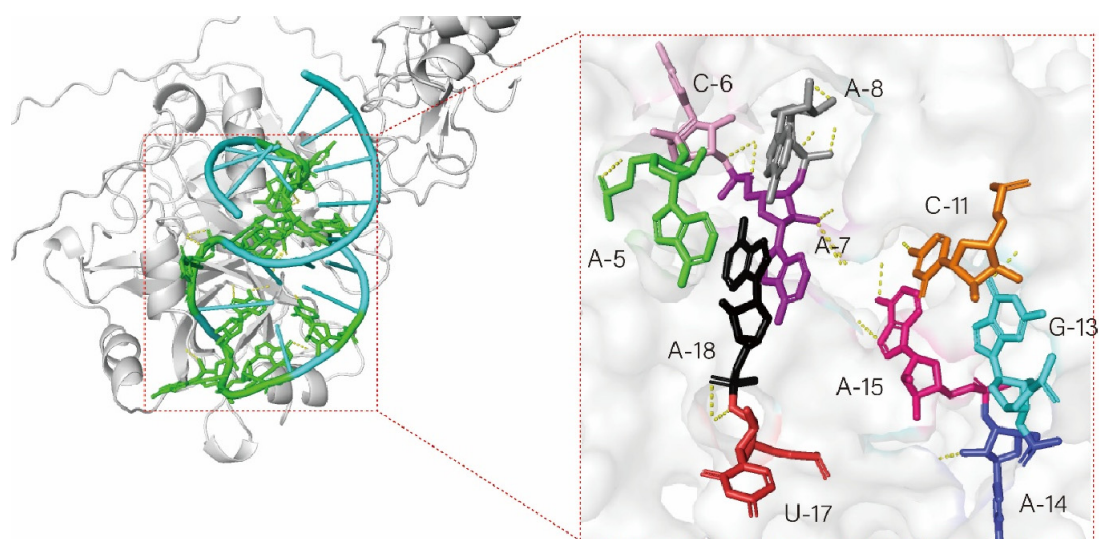
C



TBA15-Ligand



TBA29-Ligand



TBA25-Ligand

Figure S8. Candidate aptamer Mfold secondary structure prediction and AlphaFold 3 interaction structure prediction. (A) Displays the secondary structures of four candidate aptamers and their Gibbs free energy. (B) Shows the three-dimensional structure prediction of nucleic acid aptamer-ligand protein interactions using AlphaFold 3 (where the pTM score indicates the similarity between the predicted complex folding and the real structure; the pTM value greater than 0.5 indicates high similarity; the ipTM score measures the accuracy of the relative positioning of subunits within the complex, with the ipTM value above 0.8 indicating high accuracy, and below 0.6 suggesting potential inaccuracy; the color in the complex corresponds to the pIDDT value, which reflects the confidence of each atom's prediction; deeper colors represent higher pIDDT values and greater confidence). (C) Displays further molecular docking visualization of the reliable AlphaFold 3 predicted TBA15, TBA29, and TBA25 aptamers, showing the potential base sites for interaction with the ligand.

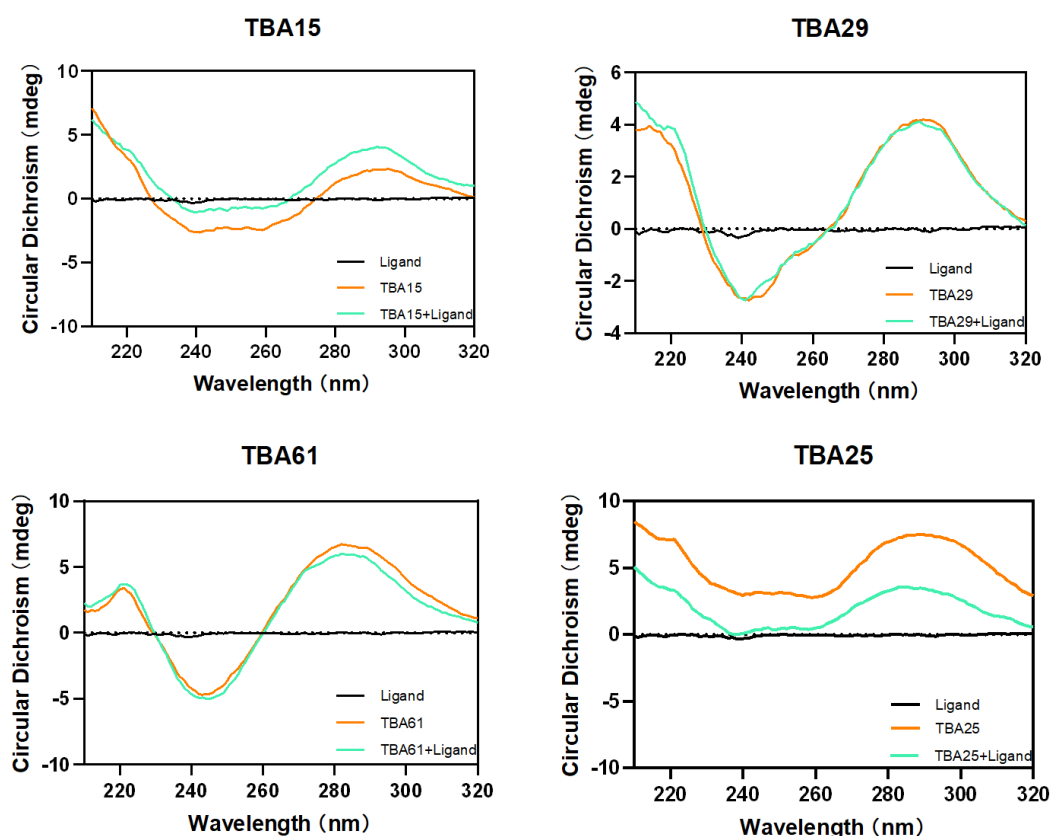


Figure S9. Circular dichroism (CD) characterization of aptamers. The figure presents the CD spectra of four candidate aptamers, with the ligand protein thrombin at a concentration of 500 nM and the aptamer at 5 μ M. The CD spectrum of the ligand alone was used as the baseline for calibration to analyze the changes induced by aptamer-ligand binding.

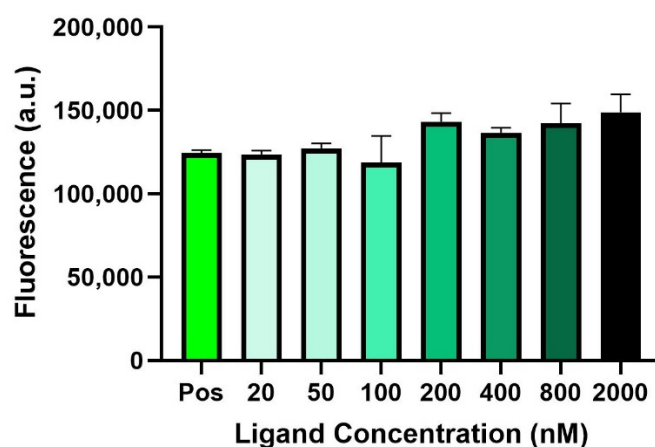


Figure S10. The effect of the ligand thrombin on *in vitro* transcription. To verify that the regulatory effect of the aptamer on transcription is due to the aptamer-ligand binding rather than the direct impact of the ligand on the transcription system, we investigated the effect of different concentrations of thrombin on the transcription system. Error bars indicate the average value of three independent biological replicates $\pm$ s.d.

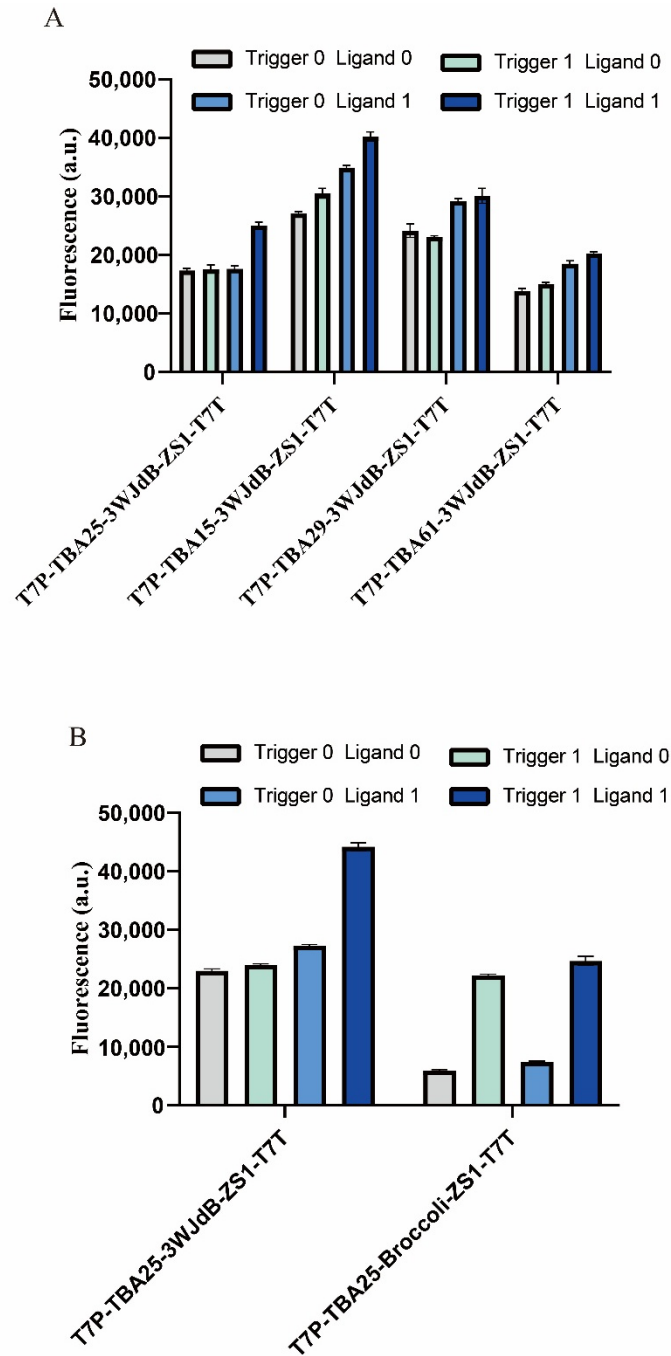


Figure S11. Investigation of APT-SWT heterogeneous input AND Gate construction. **(A)** Comparison of the regulatory potential of four candidate aptamers for AND Gate construction. **(B)** Comparison of output fluorescence when using 3WJdB and Broccoli as reporter genes. Error bars indicate the average value of three independent biological replicates $\pm$ s.d.