Supporting Information for

In vitro transcriptional regulation via nucleic-acid-based transcription factors

Leo Y.T. Chou[†‡§*] and William M. Shih[†‡*]

[†]Dana-Farber Cancer Institute. Boston, MA 02215 USA [‡]Wyss Institute of Biologically Inspired Engineering at Harvard University. Boston, MA 02215 USA [§]Present address: Institute of Biomaterials and Biomedical Engineering, University of Toronto. Toronto, ON M53 3G9 Canada.

*Correspondence should be addressed to: leo.chou@utoronto.ca and william.shih@wyss.harvard.edu

Table of Contents

Materials & Methods

Chemicals and reagents

Unless stated otherwise, all chemicals were purchased from Sigma Aldrich. All DNA oligonucleotides used in this work were purchased from Integrated DNA Technologies (IDT). The Broccoli aptamer co-factor DFHBI-1T was purchased from Lucerna Inc. (Catalog No. 410). SequaGel Urea Gel and 10x TBE Buffer for casting TBE-Urea PAGE were purchased from National Diagnostics (EC-829), and cassettes were purchased from Life Technologies (NC2010).

Cloning of N-terminal SNAP-tagged T7 RNAP

Plasmid for SNAP-tag-fused T7 RNAP was prepared by Gibson assembly of SNAP-tag gene from pSNAP-tag® (T7)-2 Vector (NEB) with linearized plasmid of T7 RNAP (pT7-911Q) using polymerase chain reaction (PCR) that introduces overlapping sequence with SNAP-tag gene using 5′-end extensions of the primers (Table S1). The assembled products were transformed into XL10-Gold Ultracompetent Cells (Agilent), and the resulting plasmid products were obtained using Plasmid Miniprep Kit (Qiagen). The plasmids were sequenced by Genewiz, Inc (South Plainfield, NJ, USA) to verify that the SNAP-tag gene was introduced correctly.

Recombinant expression of N-terminal SNAP-tagged T7 RNAP

The plasmid for SNAP-tag-fused T7 RNAP was transformed into BL21 Competent E. coli (NEB). Transformed BL21 cells were grown to OD600 around 0.5 and induced with IPTG at 0.4 mM for 3 hours. Cells were spun to pellet and re-dissolved in lysis buffer (50 mM Tris-HCl, pH 8, 300 mM NaCl, 5% glycerol, 5 mM BME), and the re-dissolved pellet was sonicated to lyse the cells. The lysate was spun down at 4^{o}C and 8,000 g to collect supernatant, and the proteins were purified

using Pierce HisPur Ni-NTA Spin Columns (Thermo Scientific) by binding the His-tagged proteins to the resin and eluting with buffer containing 200 mM imidazole to collect the purified products. Eluted proteins were buffer exchanged using Amicon Ultra filtration columns with 2x storage buffer consisting of 100 mM Tris-HCl (pH 8.0), 200 mM NaCl, 40 mM BME, 2 mM EDTA, and 0.2% Triton X-100. The SNAP-tag-fused T7 RNAP was mixed 1:1 with glycerol and stored at -20°C.

Preparation of O^6-benzylguanine-modified DNA oligonucleotides

The DNA oligonucleotides with 3′-end amine modification was purchased from IDT (Table S2). The oligonucleotides were mixed with BG-GLA-NHS (NEB), an amine-reactive substrate for SNAP-tag, to prepare O^6-benzylguanine-modified DNA oligonucleotides. BG-GLA-NHS was added in 50x molar excess to the oligonucleotides and kept above 1 mM to ensure efficient conjugation. The reaction was carried out in sodium bicarbonate buffer (50 mM, pH 8.3) and 55% DMSO at room temperature overnight. Excess BG-GLA-NHS was removed from modified oligonucleotides by 2 rounds of 1:1 v/v ethanol-acetone precipitation followed by Oligo Clean & Concentrator (Zymo Research). The concentrations of the resulting DNA oligonucleotides were measured using absorbance at 260 nm, and the oligonucleotides were stored at -20°C.

Preparation and purification of T7 RNAP-DNA conjugates

O^6-benzylguanine-modified "RPTag" oligonucleotides (Table S2) were incubated with SNAP-tag-fused T7 RNAP to form protein-DNA conjugates. O^6-benzylguanine-modified DNA oligonucleotides were added at 1.5-2x the molar concentration of SNAP-tag-fused T7 RNAP to ensure complete conjugation. Excess O^6-benzylguanine-modified DNA oligonucleotide was removed from each reaction using Pierce Strong Cation Exchange Spin Column (Thermo Scientific). The protein-DNA conjugates were eluted from the column with buffer containing 0.5 M NaCl. The eluted protein-DNA conjugates were buffer exchanged using Amicon Ultrafiltration device with 2x storage buffer (100 mM Tris-HCl (pH 8), 200 mM NaCl, 40 mM BME, 2 mM EDTA, 0.2% Triton X-100). The conjugates were mixed 1:1 with glycerol and stored at -20°C.

Synthesis of circular catenated DNA template

The circular catenated DNA template with linking number 1 was synthesized using strands in Table S3. The strands were designed to self-assemble into a nanostructure shown in Figure S6a. The oligonucleotide sequences were generated using NUPACK. All DNA strands were purchased from IDT with standard desalting option. The DNA strands were shipped dry and suspended at 0.5-2.5 mM in water and stored at -20°C. The mixture of DNA oligonucleotides (ratio indicated in Table S3 multiplied by 0.5 μM) in 1x TE + 10 mM $MgCl_2$ was subjected to a thermal annealing ramp on a Tetrad 2 Peltier Thermal Cycler (Bio-Rad) according to the following schedule: (1) Incubate at 80°C for 1 minute; (2) Decrease to 5°C @ 2 seconds/°C; (3) Store at 5°C.

1/10x volume of 10x T4 ligase buffer (NEB) and T4 ligase (NEB) at a final concentration of 0.4 unit/μL were added to the solution, and the resulting sample was ligated at room temperature

overnight. The resulting product was purified via PAGE purification to obtain the catenaned DNA template (Figure S6b).

Preparation of dsDNA templates for in vitro transcription

All oligonucleotides used for preparing dsDNA templates are purified on a 10% TBE-Urea PAGE and extracted by ethanol precipitation prior to use. Following PAGE purification, dsDNA templates for in vitro transcription were prepared in two ways. DNA templates less than 100-nt were prepared by annealing the template and non-template strands together. DNA templates longer than 100-nt were prepared by a "fill-in" reaction using a Klenow polymerase (NEB) on the template strand, and T7 promoter serving as the primer. dsDNA templates were buffer exchanged from excess components using an Oligo Clean & Concentrator spin column (Zymo Research).

Preparation of caged T7 RNAP for in vitro transcription

The dsDNA cage complex (Table S4) is annealed from 80^{o}C to 20^{o}C over 2 minutes in buffer (5 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, and 1X PBS). After annealing, the cage is combined with T7 RNAP at a stoichiometry of 5:1 in a final buffer containing 5 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, and 1X PBS. This complex is incubated at room temperature for 15 minutes before use. Two versions of the cage duplex were designed in this study; the version used in the reaction are specified in the sections below.

General in vitro transcription protocol

DNA templates and auxiliary nucleic-acid transcription factors are first mixed in buffer (5 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$) and incubated at room temperature for 20 minutes. This solution is then combined with the caged RNAP 1:1 v/v and incubated at room temperature for an additional 10 minutes. Finally, an equal volume of transcription master-mix is added to initiate transcription. The final reaction contains 1.0 mM rNTP mix (NEB), 0.4 U/μL RiboLock RNase inhibitor (Thermo Scientific), 20 μM RNA aptamer co-factor (e.g., DFHBI-1T or malachite green) in a buffer containing 40 mM Tris-HCl, 6 mM $MgCl_2$, 1 mM DTT, 2 mM spermidine, and 1X PBS. For kinetic experiments, the reaction is set up on an ice block before being transferred to a 384-well plate. The plate is assayed on a plate reader (BioTEK NEO) under 37^{o}C incubation and a reading was taken every 2 minutes over a period of 2 to 8 hours at 120 gain and 10 mm read height. For DNA templates encoding Broccoli aptamers, an excitation of 470 nm and emission of 512 nm was used; for DNA templates encoding malachite green aptamers, an excitation of 610 nm and emission of 655 nm was used.

Comparing transcriptional activity of caged (OFF) vs. uncaged (ON) RNAP

For comparing in vitro transcription using caged (OFF) vs. uncaged (ON) RNAP, dsDNA templates were prepared by thermally annealing the template and non-template strands together. For the caged (OFF) condition, an additional "blocking strand" (see Table S5) was added to the template at 10x molar excess to prevent the template from activating the RNAP. To generate the caged RNAP, ssDNA-tethered RNAP was first incubated with the cage duplex in buffer containing

5 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, and 1X PBS for 15 minutes at room temperature. The dsDNA templates were mixed with the caged RNAP, and the resulting mixture was combined 1:1 v/v with a 2X in vitro transcription master mix. The final concentration contains 1.0 mM rNTP mix (NEB), 0.4 U/μL RiboLock RNase inhibitor (Thermo Scientific), 20 μM RNA aptamer co-factor (e.g., DFHBI-1T or malachite green) in a buffer containing 40 mM Tris-HCl, 6 mM $MgCl_2$, 1 mM DTT, 2 mM spermidine. The in vitro transcription reaction was performed at 37°C in a 384-well plate and the expression of the fluorescent RNA aptamer was monitored over a period of 2 hours using a BioTEK NEO plate reader with 610 nm excitation and 655 nm emission, 120 gain and 10 mm read height. Fluorescence signals of the caged (OFF) vs. uncaged (ON) sample was compared at the end point.

For optimizing the effect of promoter sequence on the signal-to-background of the caged vs. uncaged states (e.g., shown in Figure S4B), we tested a total of six linear templates, each encoding the malachite green RNA aptamer but with different preceding promoter sequences (see Table S5; the RNA aptamer portion is highlighted in red). For comparing between caged and uncaged transcription using both linear and circular DNA templates (e.g., the results presented in Figure 1e and 1f), we used template 1 from Table S5 as the linear template, and the catenated circular DNA template described in the previous section as the circular DNA template.

Transcriptional velocity, defined as the rate of fluorescence increase per unit time, were determined by calculating the slope of the linear regions of the fluorescence kinetics curves (e.g. Figure S6). The same analysis was performed for both the linear DNA template (e.g. Figure 2e and Figure S4) and the circular DNA template (Figure 2f).

Synthetic recapitulation of the *lac* and *trp* gene regulatory architectures

To test the *lac* and *trp* gene architecture, we designed their DNA templates to encode the fluorescent Broccoli RNA aptamer as shown in Table S6 (highlighted in green). The dsDNA templates were first prepared by combining the sense and antisense strands in buffer containing 5 mM Tris, 1 mM EDTA, and 5 mM $MgCl_2$, thermally annealed at 80°C for 2 min and gradually cooled to room temperature over 30 min. The resulting dsDNA templates were then combined with repressor and effector strands in the same buffer. In a separate reaction, the ssDNA-tagged RNAP was combined with 5x molar excess of the dsDNA cage duplex in a buffer containing 5 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$ and 1x PBS, and incubated at room temperature for 15 minutes. The resulting caged RNAP was combined with the dsDNA template mixture at 1:1 v/v. A 2x transcription master mix was prepared separately and kept on ice as described in the general in vitro transcription protocol. This transcription master mix was then combined with the RNAP-template mixture at 1:1 v/v. The final transcription reaction contained 50 nM RNAP, 250 nM dsDNA cage duplex, 50 nM template, 250 nM of the repressor, and varying amounts of the effector strand (0.1x to 20x that of the repressor). The solution was then incubated at 37°C in a 384-well plate and the expression of the fluorescent RNA aptamer was monitored over a period of 2 hours using a BioTEK NEO plate reader with 470 nm excitation and 515 nm emission, 120 gain and 10 mm read height. Also note that in this experiment, we used RNA polymerase appended with a different ssDNA tether (e.g., RPTag_v2 in Table S2) and cage duplex (e.g., cage_v2 in Table S4).

Curve-fitting model for the *lac* and *trp* steady-state gene expression profiles

The steady-state fluorescence signals in Figure 3b and 3d was fit to a sigmoidal Hill equation shown below:

$$F = \frac{F_{max}[R]^h}{{K_{0.5}}^h + [R]^h}$$

Where F_0 and F_{max} are the initial and maximum fluorescence signals, respectively, *[R]* is the [Effector]:[Repressor] ratio, $K_{0.5}$ is the ratio that produces the half-maximal signal, and h is the Hill slope. For the *trp mimic*, the data was fit to a modified equation to account for the reflected shape of the curve:

$$F = F_{max}\frac{1}{1 + \frac{[R]^h}{K_{0.5}}}$$

The fitted values for the parameters are h = 6.1 and $K_{0.5}$ = 0.8 for the *Lac* system and h = 2.3 and $K_{0.5}$ = 0.085 for the *Trp* system.

Implementation of feedback and cascade systems

DNA templates for the autocatalytic and autoinhibitory systems were designed to encode the fluorescent malachite green RNA aptamer (highlighted in red) as shown in Tables S7 and S8. The dsDNA templates for auto-catalytic and auto-inhibitory feedback templates were set up in the same way as the previous section. For the autocatalytic system, the trigger template and autocatalytic template were annealed separately and then combined. The Repressor strand was combined with the templates post-annealing, then mixed with caged RNAP as described in previous section. The final in vitro transcription reaction contained 50 nM RNAP, 250 nM dsDNA cage, 125 nM template, and varying amounts of repressor strand as noted in Figures 4b and 4d. For the auto-catalytic template, the final concentration of the initial trigger template was 1 nM. The solution was incubated at 37^{o}C in a 384-well plate and the expression of a fluorescent RNA aptamer was monitored over the course of 4 to 8 hours using a BioTEK NEO plate reader with excitation at 610 nm and emission at 655 nm. To reduce the noise in the time series data, the fluorescence vs. time traces shown in Figure 4b & 4d was fit to a cubic interpolating spline using the UnivariateSpline function from the Scipy package in Python with parameters k = 3 and w = 1/σ (y), where σ (y) is the standard deviation in the moving average of the fluorescence signal. This fit is plotted as dotted lines in the same graph as the kinetic traces shown as individual markers to help guide the viewer.

Implementation of transcriptional multiplexing

DNA strands were ordered as 96-well plate oligos from IDT (Table S9). Strands were reconstituted in water and concentration was measured using Nanodrop (Thermo Fisher). Each template was separately annealed in buffer containing 5 mM Tris, 1 mM EDTA, and 5 mM $MgCl_2$. Annealed templates were pooled together in equal volumes to generate a master template pool. The pooled template was split into 12 aliquots, each mixed with a respective pair of TF strands and the caged

RNAP solution prepared as described above. The mixture was then combined with a 2X transcription master mix and incubated at 37°C for 2 hours. The final solution contained a total template concentration of 600 nM (each template at 50 nM), 100 nM of the TF pair, 40 nM RNAP, and 400 nM dsDNA cage. After transcription, 5.0 µl of DNase I (2 U/µl, NEB) was added to the reaction and incubated at 37°C for additional 30 min to remove the DNA templates. Transcripts were purified using an RNA Oligo Clean & Concentrator (Zymo) and yield was quantified using Nanodrop. 1 µL of the RNA transcript before and after DNase I treatment were aliquoted for denaturing PAGE analysis. The concentration of the purified RNA was quantified by measuring absorbance at 260 nm. Purified RNA transcripts were stored at -80°C before use.

Identification of transcripts from multiplexed transcription using molecular beacons

The overall architecture of the beacon is shown in Figure S10, and consists of four strands, P, C, Q, and F. The Q and F strands are universal, while the P and C strands are unique to each template (see Table S10). Molecular beacons were prepared by combining these strands in buffer containing 5 mM Tris, 1 mM EDTA, and 5 mM $MgCl_2$. The solution was then annealed at 80°C for 2 min and cooled to temperature over 20 min. The final concentration of the solution contained 10 nM C strand, 30 nM P strand, 10 nM F strand, 50 nM Q strand.

To assay RNA transcripts, we first normalized the concentration of all RNA transcripts to 50 nM. Then, 23.5 µl of each beacon solution was added to 1.5 µl of each purified RNA transcript. The solution was incubated at 37°C for 1 h in 384-well plates and the fluorescence was read using the BioTEK NEO plate reader using the following setting: 610 nm excitation / 660 nm emission; 120 gain; 10 mm height. Positive controls were included by combining 23.5 µl of the molecular beacons with 1.5 µl of 100 µM sense template. Negative controls were included by combining 23.5 µl of the molecular beacons with buffer.

Supporting Tables

Table S1. List of primer sequence for PCR of T7 RNAP

Name	Sequence
pT7-911Q forward primer	TGGGTAAACCGGGTCTGGGAGGATCCATGAACACGATTAA
pT7-911Q reverse primer	TTCATTTCGCAATCTTTGTCGTGATGGTGATGGTGATGCG

Table S2. List of DNA oligonucleotides with 3′ amine-modifications for tethering to RNAP

Name	Sequence
RPTag_v1	GCTACTCACTCAGATAGGGTA/3AmMO/
RPTag_v2	AATTAGGATTTCATGTCAGGAGATTTTCAGCC/3AmMO/

Table S3. List of DNA oligonucleotides for generating catenated circular DNA template

Name	Sequence	Molar Ratio
DNA leash	/5Phos/GGTTTACCCTATCTGAGTGAGTAGCGGAGTAG AGTGGAACCCTAACCTAATTATAGAGAA	4
DNA template	/5Phos/GCGGAGCCCACACTCTACTCGACAGATACGA ATATCTGGACCCGACCGTCTCCGCCATCCCCACACG CGTGGGGTCTGCCTATAGTGAGTCGTATTA	1
DNA template splint	TTAGGCAGACCCCACGCGTGTGGGGATGTGGGCTCC GCTAATACGACTCACTATTT	2
Catenane adaptor1	TCGTATCTGT	5
Catenane adaptor2	GCTACTCACT	5
Catenane adaptor3	GTTAGGGTTC	5
DNA leash splint	AGGGTAAACCTTCTC	5

** Bases colored in green denote the Broccoli aptamer*

Table S4. List of DNA oligonucleotides used to generate the dsDNA cage

Name	Sequence
Cage_v1 sense strand	TACCCTATCTGAGTT TT CAAATTAATACGACTCACTATA
Cage_v1 antisense strand	TATAGTGAGTCGTATTAATTTG
Cage_v2 sense strand	GGCTGAAAATCTCCT TT GAAATTAATACGACTCACTATA
Cage_v2 antisense strand	TATAGTGAGTCGTATTAATTTC

Table S5. List of DNA oligonucleotides used for testing transcriptional activation

Name	Sequence
Temp 1 (sense)	TAAGACGACTCACTATAGGGATCCCGACTGGCGAGAGCCAGGTA ACGAATGGATCC

Temp 2 (sense)	TAATTGAACTCACTAAAGGGATCCCGACTGGCGAGAGCCAGGTA ACGAATGGATCC
Temp 3 (sense)	TAATACGTCTCACTATAGGGATCCCGACTGGCGAGAGCCAGGTA ACGAATGGATCC
Temp 4 (sense)	TAATACGACTCTCTATAGGGATCCCGACTGGCGAGAGCCAGGTA ACGAATGGATCC
Temp 5 (sense)	TAATACGACTCAGTATAGGGATCCCGACTGGCGAGAGCCAGGTA ACGAATGGATCC
Temp 6 (sense)	TATTACGACTCACTAAAGGGATCCCGACTGGCGAGAGCCAGGTA ACGAATGGATCC
Temp 1 (antisense)	GGATCCATTCGTTACCTGGCTCTCGCCAGTCGGGATCCCTATAGT GAGTCGTCTTATACCCTATCTGAGTGAGGTAG
Temp 2 (antisense)	GGATCCATTCGTTACCTGGCTCTCGCCAGTCGGGATCCCTTTAGT GAGTTCAATTATACCCTATCTGAGTGAGGTAG
Temp 3 (antisense)	GGATCCATTCGTTACCTGGCTCTCGCCAGTCGGGATCCCTATAGT GAGACGTATTATACCCTATCTGAGTGAGGTAG
Temp 4 (antisense)	GGATCCATTCGTTACCTGGCTCTCGCCAGTCGGGATCCCTATAGA GAGTCGTATTATACCCTATCTGAGTGAGGTAG
Temp 5 (antisense)	GGATCCATTCGTTACCTGGCTCTCGCCAGTCGGGATCCCTATACT GAGTCGTATTATACCCTATCTGAGTGAGGTAG
Temp 6 (antisense)	GGATCCATTCGTTACCTGGCTCTCGCCAGTCGGGATCCCTTTAGT GAGTCGTAATATACCCTATCTGAGTGAGGTAG
Blocking strand (linear)	CTACCTCACTCAGATAGGGTA
Blocking strand (circular)	GCTACTCACTCAGATAGGGTA

* *Bases colored in red denote the Malachite Green aptamer*

Table S6. List of DNA oligonucleotides for constructing the lac and trp mimics

Name	**Sequence**
Lac template (sense)	TAATACGACTCACTATAGGGAGAGTGGGAGACGGTCGGGTCC AGATATTCGTATCTGTCGAGTAGAGTGTGGGCTCCCAC
Lac Template (antisense)	GTGGGAGCCCACACTCTACTCGACAGATACGAATATCTGGAC CCGACCGTCTCCCACTCTCCCTATAGTGAGTCGTATTACTGAA AATCTCCTGACATGCGCTACCATA
Lac repressor	CAACGATGCTTATGGTAGCGCATGTCAG
Lac effector	CGCTACCATAAGCATCGTTG
Lac effectorΔ1	CGCTACCATAAGCATCGTT
Lac effectorΔ2	CGCTACCATAAGCATCGT
Lac effectorΔ3	CGCTACCATAAGCATCG

Lac effectorΔ4	CGCTACCATAAGCATC
Trp template (sense)	TAATACGACTCACTATAGGGAGAGTGGGAGACGGTCGGGTCCAGATATTCGTATCTGTCGAGTAGAGTGTGGGCTCCCAC
Trp Template (antisense)	GTGGGAGCCCACACTCTACTCGACAGATACGAATATCTGGACCCGACCGTCTCCCACTCTCCCTATAGTGAGTCGTATTACTGAAAATCTCCTGACATGCGCTACCATA
Trp 4WJ repressor	CATTGCCACGAAGAT GGATACTCCTGTATCC CATGTCAG
Trp 4WJ effector	TATGGTAGCG ATCTTCGTGGCAATG
Trp 4WJ effectorΔ2	TGGTAGCG ATCTTCGTGGCAATG
Trp 4WJ effectorΔ3	GGTAGCG ATCTTCGTGGCAATG
Trp 4WJ effectorΔ4	GTAGCG ATCTTCGTGGCAATG
Trp 4WJ effectorΔ5	TAGCG ATCTTCGTGGCAATG
Trp 3WJ repressor	CTCCAACGATGCTCATGTCAG
Trp 3WJ effector	TATGGTAGCGAGCATCGTTGGAG

** Bases colored in green denote the Broccoli aptamer*

Table S7. List of DNA oligonucleotides for the autoinhibitory feedback circuit in Fig. 3B

Name	**Sequence***
Autoinhibitory template (sense)	TAATACGACTCACTATAGGGATCCCGACTGGCGAGAGCCAGGTAACGAATGGATCCCAGGTACTGCTAGACTTCGTGGCAATC
Autoinhibitory template (antisense)	GATTGCCACGAAGTCTAGCAGTACCTGGGATCCATTCGTTACCTGGCTCTCGCCAGTCGGGATCCCTATAGTGAGTCGTATTACTATCTGAGTGAGTAGCAGTACCTG
Repressor	GATTGCCACGAAGTCGGATACTCCTGTATCCCTCACTCAGAT

** Bases colored in red denote the Malachite Green aptamer*

Table S8. List of DNA oligonucleotides for the autocatalytic feedback circuit in Fig. 3D

Name	**Sequence**
Autocatalytic template (sense)	TAATACGACTCACTATAGGGATCCCGACTGGCGAGAGCCAGGTAACGAATGGATCCGCCGCTACCATAAGCATCGTTGAG
Autocatalytic template (antisense)	CTCAACGATGCTTATGGTAGCGGCGGATCCATTCGTTACCTGGCTCTCGCCAGTCGGGATCCCTATAGTGAGTCGTATTACCTATCTGAGTGAGTAGCCGCTACCATA
Trigger template (sense)	TAATACGACTCACTATAGGGAGAGCCGCTACCATAAGCATCGTTGAG
Trigger template (antisense)	CTCAACGATGCTTATGGTAGCGGCTCTCCCTATAGTGAGTCGTATTACCTATCTGAGTGAGTAGCCTCCACATCCCATTACCACTCCCAC
Repressor	CTCAACGATGCTTATGGTAGCGGCTACTCACT

** Bases colored in red denote the Malachite Green aptamer*

Table S9. List of DNA oligonucleotides for implementing transcriptional multiplexing

Template (sense) 1	TAATACGACTCACTATAGGGAGATGAGGATAGAGGCGAGTGAATAGGATT
Template (sense) 2	TAATACGACTCACTATAGGGAGATTAAGGTGGAAGTAGGTAGGAGTTGTT
Template (sense) 3	TAATACGACTCACTATAGGGAGATGACGACGAATAACACTAATACAGCTT
Template (sense) 4	TAATACGACTCACTATAGGGAGATGAAGTGAACGAGGATAGATAAGGATT
Template (sense) 5	TAATACGACTCACTATAGGGAGATGAGGTGAGGATGATGGGAGTGAAATT
Template (sense) 6	TAATACGACTCACTATAGGGAGATGAAATGATGATGGATAGAACTGGATT
Template (sense) 7	TAATACGACTCACTATAGGGAGATACTTACTTACTACGCAACTTATACTT
Template (sense) 8	TAATACGACTCACTATAGGGAGACTACCACTGCCTATATCACGACCTTTT
Template (sense) 9	TAATACGACTCACTATAGGGAGACTGATATACTACTGACTACTTGAAATT
Template (sense) 10	TAATACGACTCACTATAGGGAGATGGGACGGGATGAGATGATAGAGCTTT
Template (sense) 11	TAATACGACTCACTATAGGGAGATGAGTGAGGTGGAGTGAGGGTAGGGTT
Template (sense) 12	TAATACGACTCACTATAGGGAGATAAGAACACTAAGGATGAATAACGATT
Template (antisense) 1	AATCCTATTCACTCGCCTCTATCCTCATCTCCCTATAGTGAGTCGTATTACAGTTCCATTATCGCCGTAGTTGGTGTACT
Template (antisense) 2	AACAACTCCTACCTACTTCCACCTTAATCTCCCTATAGTGAGTCGTATTAAGCAATCTGAGAAGGTGACTCTCTACTGCT
Template (antisense) 3	AAGCTGTATTAGTGTTATTCGTCGTCATCTCCCTATAGTGAGTCGTATTAAGCTTGTCACTAGTGGCTGGTCCTACTTAG
Template (antisense) 4	AATCCTTATCTATCCTCGTTCACTTCATCTCCCTATAGTGAGTCGTATTAGGTGGTAATCGTCTTGTTGTCGACATCAAG
Template (antisense) 5	AATTTCACTCCCATCATCCTCACCTCATCTCCCTATAGTGAGTCGTATTACGAGTATTGATTCGCGAGCTCATGAAGACA
Template (antisense) 6	AATCCAGTTCTATCCATCATCATTTCATCTCCCTATAGTGAGTCGTATTAGGCTACTCTTCCATCGGCTAGATCCAGAAC
Template (antisense) 7	AAGTATAAGTTGCGTAGTAAGTAAGTATCTCCCTATAGTGAGTCGTATTACGAAGTTCTCACCTTGCACTTAGGAGACTC
Template (antisense) 8	AAAAGGTCGTGATATAGGCAGTGGTAGTCTCCCTATAGTGAGTCGTATTACTCTTGGACGTAAGGCGATTGGATTGAGGA
Template (antisense) 9	AATTTCAAGTAGTCAGTAGTATATCAGTCTCCCTATAGTGAGTCGTATTAGCCACATTCAATTGGGCTGTCTTAGCAAGT
Template (antisense) 10	AAAGCTCTATCATCTCATCCCGTCCCATCTCCCTATAGTGAGTCGTATTAACTGTAACAACAGGCTCTTGATGACAGTCG

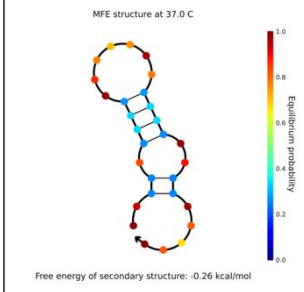
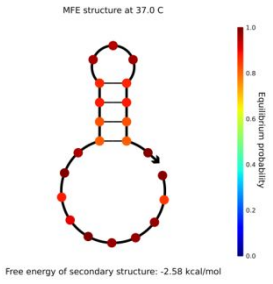
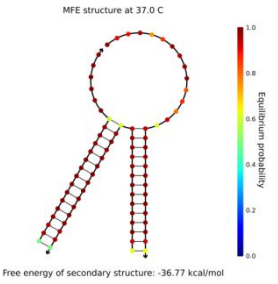
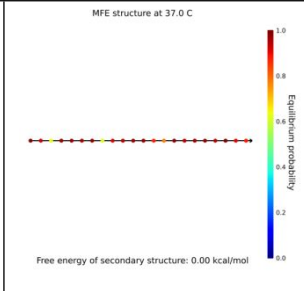
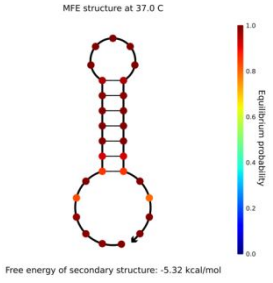
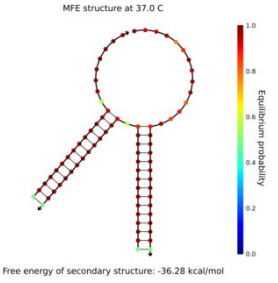
Template (antisense) 11	AACCCTACCCTCACTCCACCTCACTCATCTCCCTATAGTGAGTCGT ATTACCAACGTATCAGCTTGATTCTTGAGAGGCC
Template (antisense) 12	AATCGTTATTCATCCTTAGTGTTCTTATCTCCCTATAGTGAGTCGTA TTACGGAAGTCAGACTTCCGCATATACTCGTGA
TF_A_1	AGTACACCAACTACGGAGTAGC
TF_A_2	AGCAGTAGAGAGTCAGAGTAGC
TF_A_3	CTAAGTAGGACCAGCGAGTAGC
TF_A_4	CTTGATGTCGACAACGAGTAGC
TF_A_5	TGTCTTCATGAGCTCGAGTAGC
TF_A_6	GTTCTGGATCTAGCCGAGTAGC
TF_A_7	GAGTCTCCTAAGTGCGAGTAGC
TF_A_8	TCCTCAATCCAATCGGAGTAGC
TF_A_9	ACTTGCTAAGACAGCGAGTAGC
TF_A_10	CGACTGTCATCAAGAGAGTAGC
TF_A_11	GGCCTCTCAAGAATCGAGTAGC
TF_A_12	TCACGAGTATATGCGGAGTAGC
TF_B_1	TACCCTATCTGAGTGCGATAATGGAACTG
TF_B_2	TACCCTATCTGAGTCCTTCTCAGATTGCT
TF_B_3	TACCCTATCTGAGTCACTAGTGACAAGCT
TF_B_4	TACCCTATCTGAGTAAGACGATTACCACC
TF_B_5	TACCCTATCTGAGTGCGAATCAATACTCG
TF_B_6	TACCCTATCTGAGTGATGGAAGAGTAGCC
TF_B_7	TACCCTATCTGAGTAAGGTGAGAACTTCG
TF_B_8	TACCCTATCTGAGTCCTTACGTCCAAGAG
TF_B_9	TACCCTATCTGAGTCCAATTGAATGTGGC
TF_B_10	TACCCTATCTGAGTGCCTGTTGTTACAGT
TF_B_11	TACCCTATCTGAGTAAGCTGATACGTTGG
TF_B_12	TACCCTATCTGAGTGAAGTCTGACTTCCG

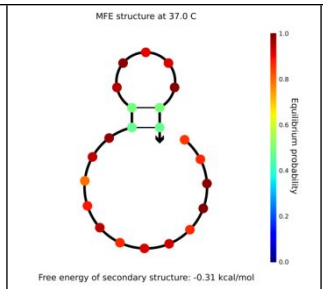
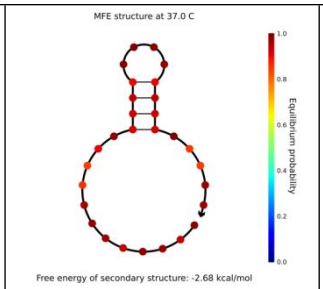
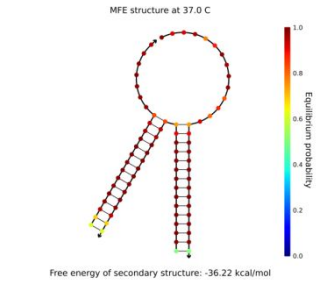
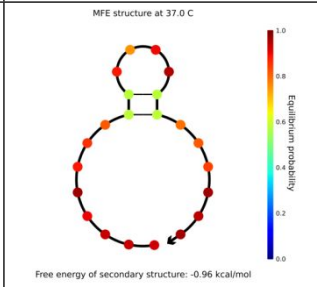
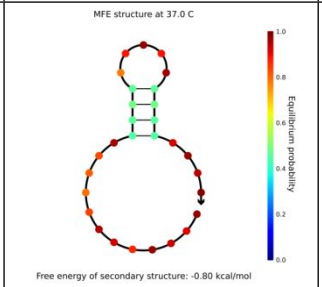
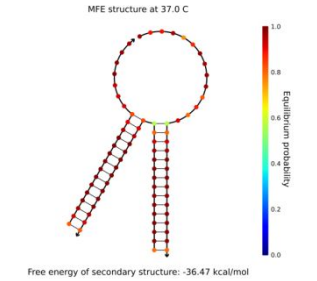
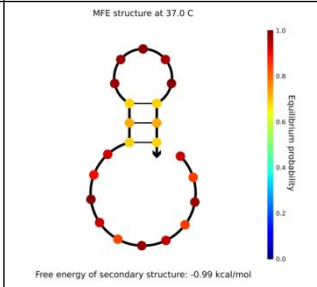
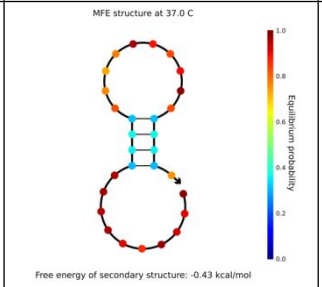
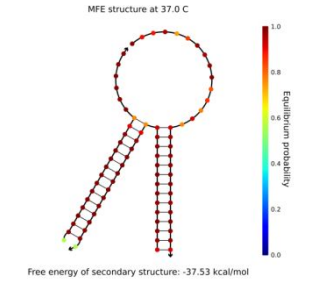
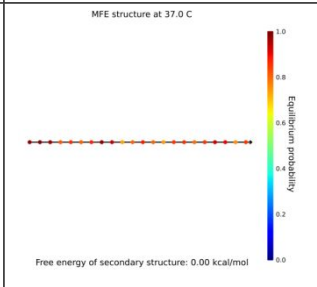
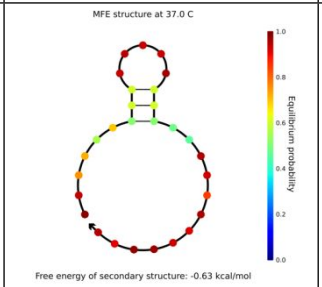
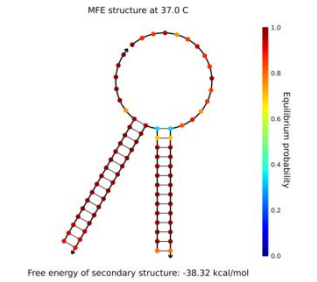
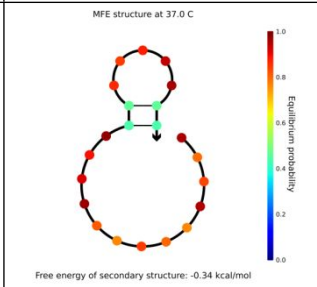
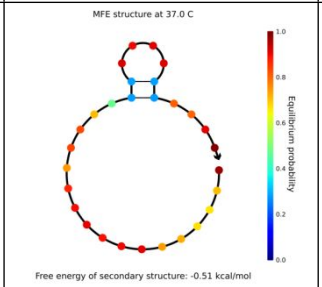
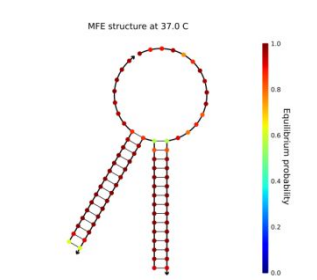
Table S10. List of DNA oligonucleotides for constructing molecular beacons

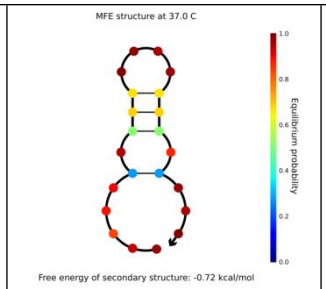
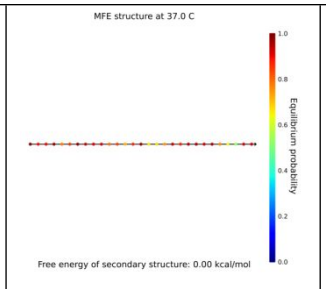
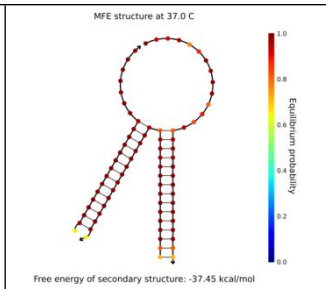
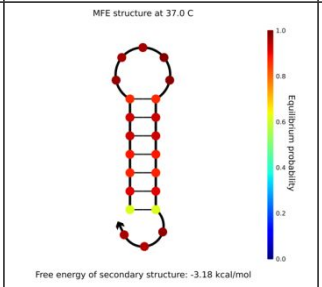
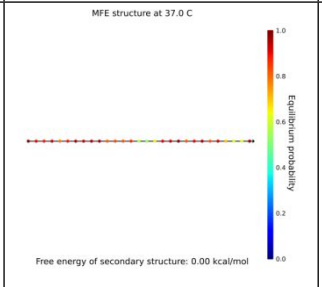
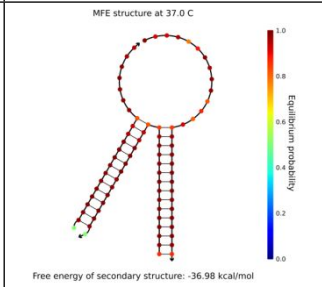
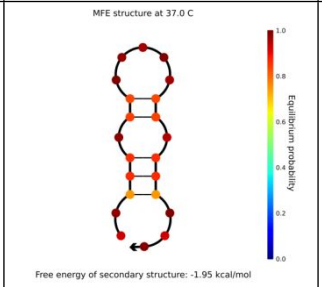
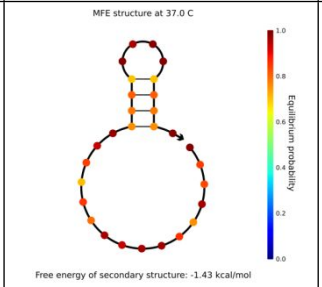
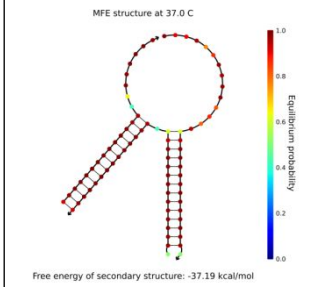
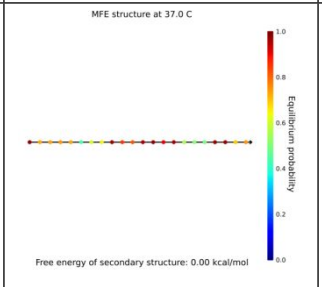
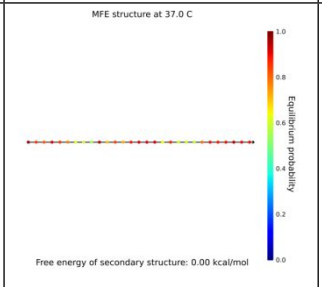
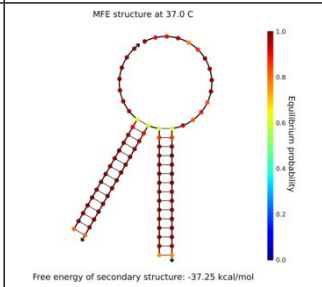
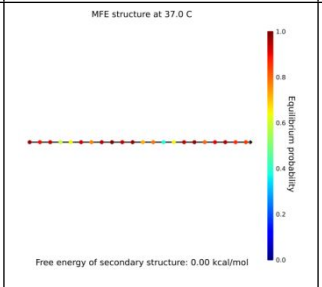
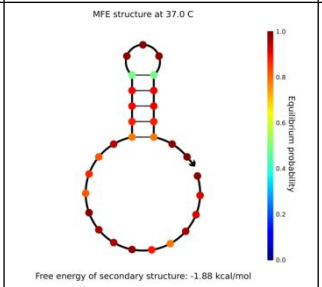
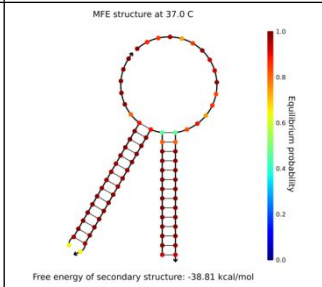
Name	**Sequence**
P1	AAGGACGAGCAAATGTACCTGCATGAGGATAGAGGCGAGTG
P2	AAGGACGAGCAAATGTACCTGCATTAAGGTGGAAGTAGGTA
P3	AAGGACGAGCAAATGTACCTGCATGACGACGAATAACACTA
P4	AAGGACGAGCAAATGTACCTGCATGAAGTGAACGAGGATAG
P5	AAGGACGAGCAAATGTACCTGCATGAGGTGAGGATGATGGG
P6	AAGGACGAGCAAATGTACCTGCATGAAATGATGATGGATAG
P7	AAGGACGAGCAAATGTACCTGCATACTTACTTACTACGCAA
P8	AAGGACGAGCAAATGTACCTGCACTACCACTGCCTATATCA
P9	AAGGACGAGCAAATGTACCTGCACTGATATACTACTGACTA

P10	AAGGACGAGCAAATGTACCTGCATGGGACGGGATGAGATGA
P11	AAGGACGAGCAAATGTACCTGCATGAGTGAGGTGGAGTGAG
P12	AAGGACGAGCAAATGTACCTGCATAAGAACACTAAGGATGA
C1	TCCTATTCACTCGCCTCTATCCTCATGGTCTACTATCCACGATTTAAC
C2	CAACTCCTACCTACTTCCACCTTAATGGTCTACTATCCACGATTTAAC
C3	GCTGTATTAGTGTTATTCGTCGTCATGGTCTACTATCCACGATTTAAC
C4	TCCTTATCTATCCTCGTTCACTTCATGGTCTACTATCCACGATTTAAC
C5	TTTCACTCCCATCATCCTCACCTCATGGTCTACTATCCACGATTTAAC
C6	TCCAGTTCTATCCATCATCATTTCATGGTCTACTATCCACGATTTAAC
C7	GTATAAGTTGCGTAGTAAGTAAGTATGGTCTACTATCCACGATTTAAC
C8	AAGGTCGTGATATAGGCAGTGGTAGTGGTCTACTATCCACGATTTAAC
C9	TTTCAAGTAGTCAGTAGTATATCAGTGGTCTACTATCCACGATTTAAC
C10	AGCTCTATCATCTCATCCCGTCCCATGGTCTACTATCCACGATTTAAC
C11	CCCTACCCTCACTCCACCTCACTCATGGTCTACTATCCACGATTTAAC
C12	TCGTTATTCATCCTTAGTGTTCTTATGGTCTACTATCCACGATTTAAC
Q strand	/5IAbRQ/GT GCG AAC AGG TAC ATT TGC TCG TCC TT
F strand	GTT AAA TCG TGG ATA GTA GAC TTC GCA C/3Cy5sp/

Table S11 NUPACK predicted secondary structures and thermodynamic properties of the TF pairs shown in Figure 5

TF Pair	SS* [TF_A]	SS [TF_B]	SS [Template**-TF_A-TF_B]	Eq. Conc.***
1				48 nM (96%)
2				28 nM (56%)

3				43 nM (86%)
4				49 nM (98%)
5				47 nM (94%)
6				44 nM (88%)
7				48 nM (96%)

8				50 nM (100%)
9				29 nM (58%)
10				45 nM (90%)
11				50 nM (100%)
12				46 nM (92%)

N.B. structures are calculated using NuPACK with the following parameters: [DNA template] = 50 nM; [TF_A] = [TF_B] = 100nM; [Temperature] = 37^{o}C; [$MgCl_2$] = 6 mM; [NaCl] = 150 mM.
** SS = secondary structure*

*** Only the ssDNA operating domain of the template was used as the input in NuPACK*
**** Equilibrium concentration of the [Template*-TF_A-TF_B] complex. Numbers in parentheses denote the percentage of template bound as ternary complex out of total template concentration.*

Table S12. Fluorescence values for the heat map shown in Figure 5.

Beacon \ Transcript	1	2	3	4	5	6	7	8	9	10	11	12	Neg Ctrl*
1	188	34	20	39	44	31	38	25	21	46	28	49	36
2	65	516	18	31	25	51	33	42	34	46	66	38	64
3	44	42	229	42	23	48	67	56	52	49	18	33	71
4	26	42	52	236	34	31	34	39	24	41	33	32	47
5	39	32	42	41	485	53	50	39	32	49	48	25	28
6	36	50	44	65	48	498	41	63	41	71	52	43	39
7	49	43	50	74	33	44	151	55	83	77	52	44	70
8	26	38	38	24	38	44	29	382	56	79	55	40	56
9	38	74	26	58	41	68	56	69	215	76	35	49	48
10	18	43	39	37	47	42	49	25	40	509	40	41	42
11	58	64	25	28	67	45	35	36	40	64	303	33	41
12	34	49	64	56	40	42	59	68	89	50	39	424	71

** Columns labeled 1 to 12 corresponds to RNA produced from one of the twelve in vitro transcription reactions. Negative control column contained 1x PBS with no RNA*

Supporting Figures

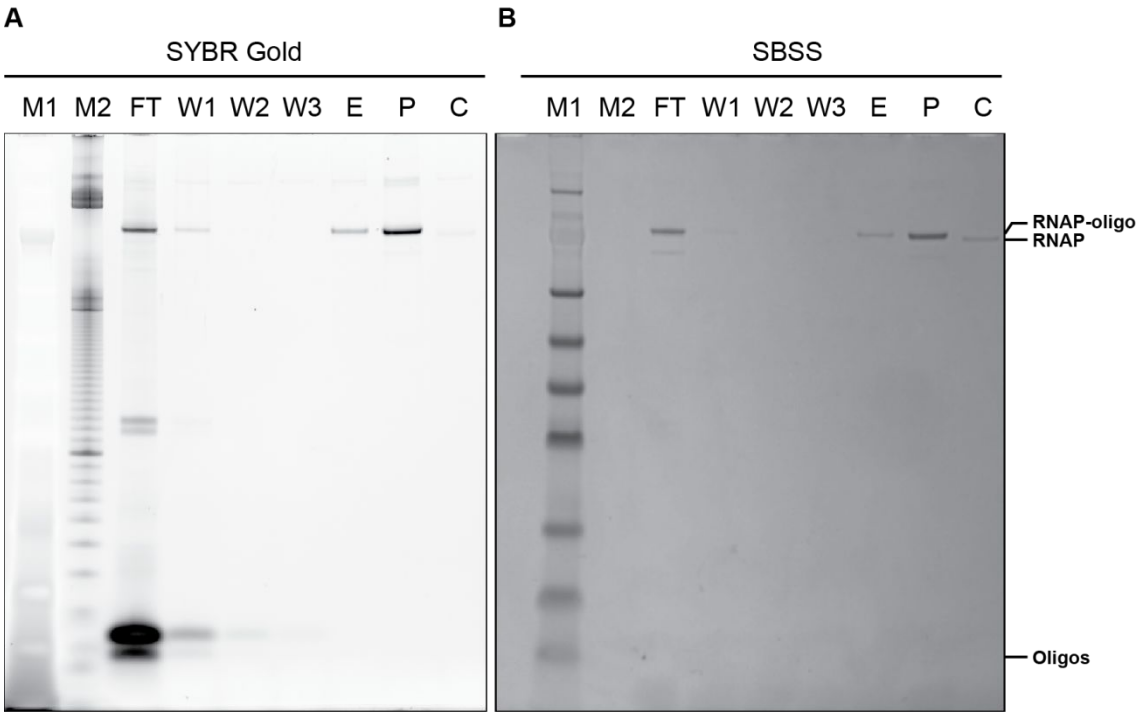


Figure S1. SDS-PAGE analysis of T7 RNAP-oligonucleotide conjugation and purification. RNAP-oligonucleotide conjugates were eluted from the crude reaction using a strong cation exchange spin column (catalog number: 90008 from Thermo Fisher) and analyzed on SDS-PAGE following (A) SYBR™ Gold stain and (B) SimplyBlue™ SafeStain. M1 = protein ladder (SeeBlue™ Plus from Thermo Fisher), M2 = DNA ladder (10-bp ladder from Thermo Fisher). FT = flow through from the column, W1-W3 = wash fractions showing removal of excess unreacted oligonucleotides, E = elution fractions showing generation of pure protein-oligonucleotide conjugates, P = elution + amicon filtration showing up-concentration of the conjugates, C = control containing the unreacted SNAP-tagged RNAP showing the slight mobility shift following conjugation.

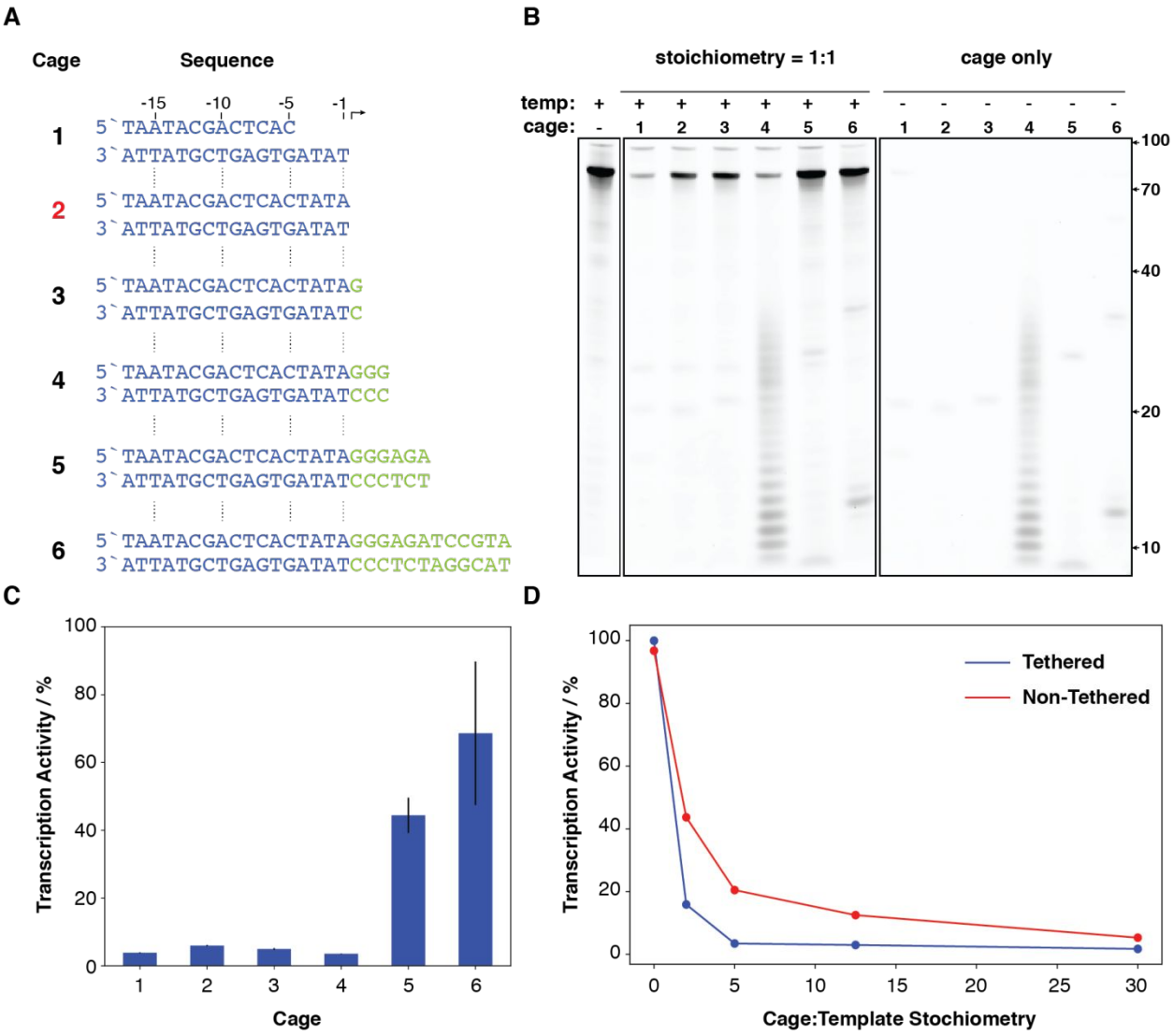


Figure S2. Proposed mechanism of T7 RNAP caging. (A) Six dsDNA cage designs were assayed for RNAP inhibition during in vitro transcription reactions. Promoter sequences are colored blue and transcript sequence in green. The design chosen for subsequent experiments is highlighted in red. (B) Denaturing TBE-Urea PAGE showing in vitro transcription products using the DNA template alone (left), DNA template mixed with various cage designs at 1:1 stoichiometry (middle), or the cages alone (right). (C) Quantification of caging efficiency. Transcriptional activity is defined as the band intensity of the RNA transcript in Panel B normalized to that of the template-only lane as a control. (D) Effect of adding a tethering domain to the dsDNA cage on its ability to inhibit RNAP activity at different stoichiometry. These results show that when the cage encodes transcripts that are 6-nt or longer, it functions as a stochiometric competitor (e.g. reducing transcription efficiency by ~50% when added at 1:1 stoichiometry relative to the template). On the other hand, when the cage encoded transcripts less than 6-nt long (i.e. Designs 1-4), it inhibits transcription initiation. We hypothesize that these designs function by binding to the RNAP and preventing the ability of the polymerase to associate with other DNA template present in solution. This hypothesis is consistent with the highest inhibition efficiency being observed for Design 1, in which the last 4-nt in the sense strand of the T7 promoter sequence is omitted, which is known to further strengthen RNAP-promoter interaction and reduce promoter escape efficiency.

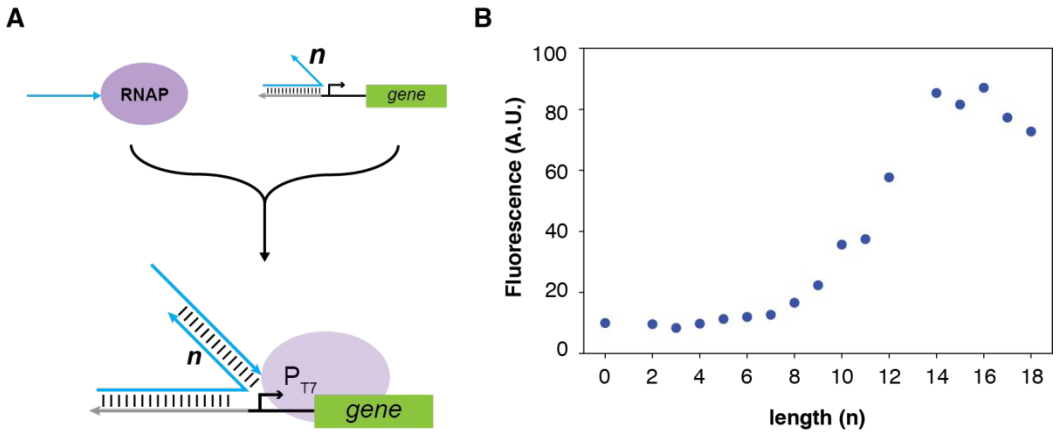


Figure S3. Tuning transcriptional strength using the length of the cis-regulatory domain. (A) Schematic of the experiment. DNA-tagged RNAP is "loaded" onto its linear DNA template via an adapter sequence (light blue), which contains a complementary region to the DNA-tagged RNAP of variable length, labeled ***n***. Changing the length of this domain modulates the stability of RNAP binding to its DNA template. The downstream gene (light green rectangle) encodes for a fluorescent RNA aptamer whose expression can be monitored in real time. (B) Relative amounts of the fluorescent RNA aptamer produced after 30 minutes of in vitro transcription as a function of the domain length (***n***) shown in panel A. Increasing domain length results in an increase in the amount of RNA transcripts produced up to a saturation point at n = 15 nt. Beyond this point there is a slight decrease in transcriptional activity. We hypothesize that saturation occurs as result of stable binding between the RNAP and its DNA template. Below this point, spontaneous dissociation between the RNAP and template can occur. In contrast, beyond the saturation point, additional increase in the length ***n*** does not further reduce spontaneous dissociation, but rather can inhibit RNAP transcription by reducing its mobility. The length-dependent transcriptional activation provides a straightforward strategy to tune the transcriptional strength of a gene by controlling RNAP binding stability to the gene.

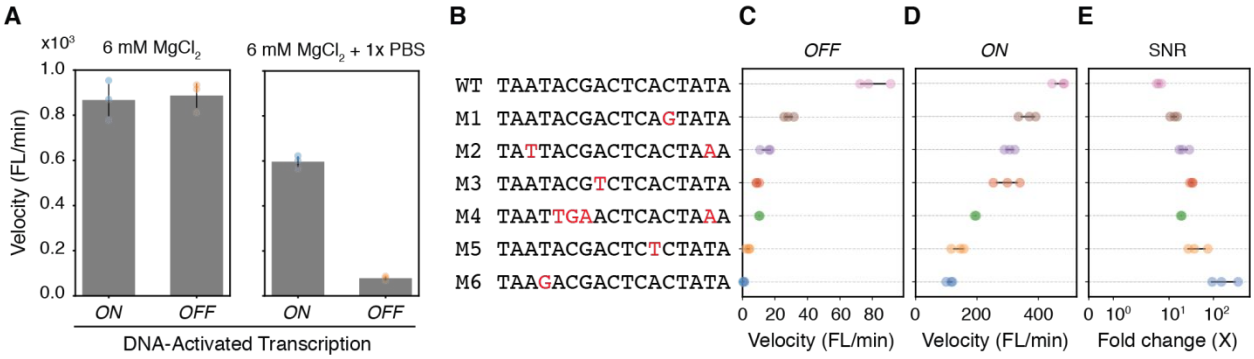


Figure S4. Optimization of transcriptional activation signal-to-background. (A) Comparison of transcriptional velocity in buffers containing different ions. Addition of 1X phosphate buffered saline (PBS) significantly decreases transcription in the ON and OFF states by 1.4-fold and 10-fold, respectively, resulting in increased signal-to-background ratio (B) Comparison of gene expression under regulation by seven different T7 promoters. Letters highlighted in red are the mutations in reference to the wild-type (WT) T7 promoter sequence. (C) Transcription in the OFF state, (D) transcription in the ON state, (E) Signal-to-background ratio, determined as the ratio of the transcription velocity between the ON and OFF states.

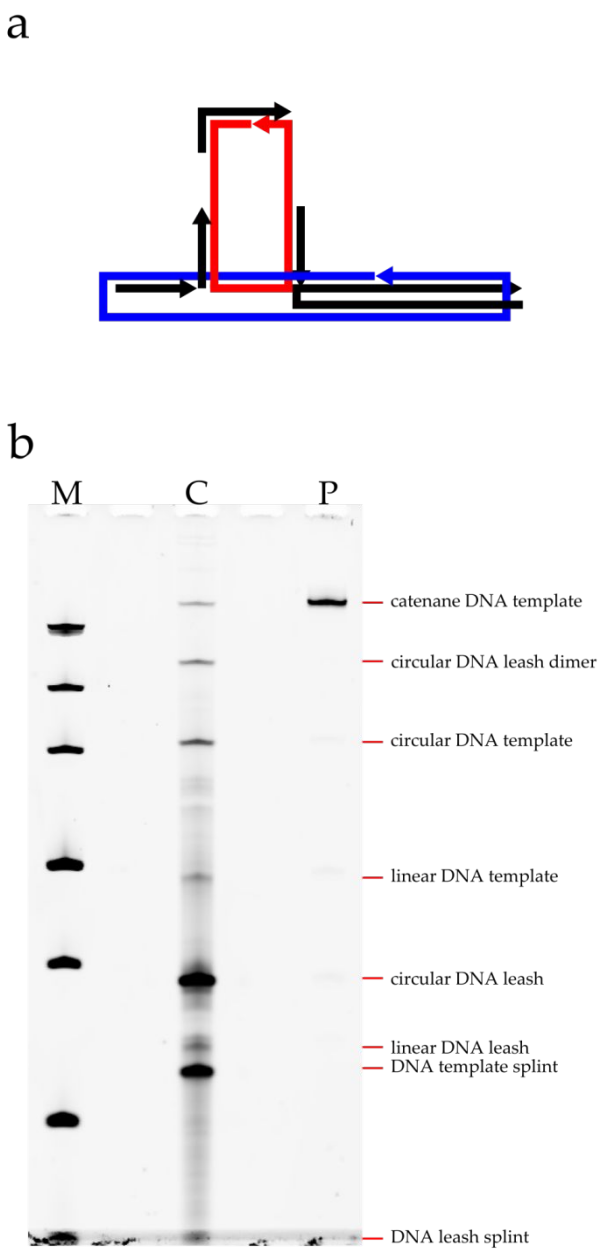


Figure S5. Synthesis of catenane DNA template. (a) Illustration of nanostructure architecture for synthesis of catenane DNA template. DNA leash (red), template (blue), and splint/adaptor (black) strands assemble together through hybridization, and DNA leash and template strands are mechanically locked to one another following the ligation reaction that circularizes each strand. Line and arrowhead represent ssDNA and 3′-end, respectively. (b) PAGE analysis of catenane DNA template. M = Ultra Low Range DNA Ladder, C = crude ligation reaction for synthesis of catenane DNA template, P = PAGE-purified catenane DNA template.

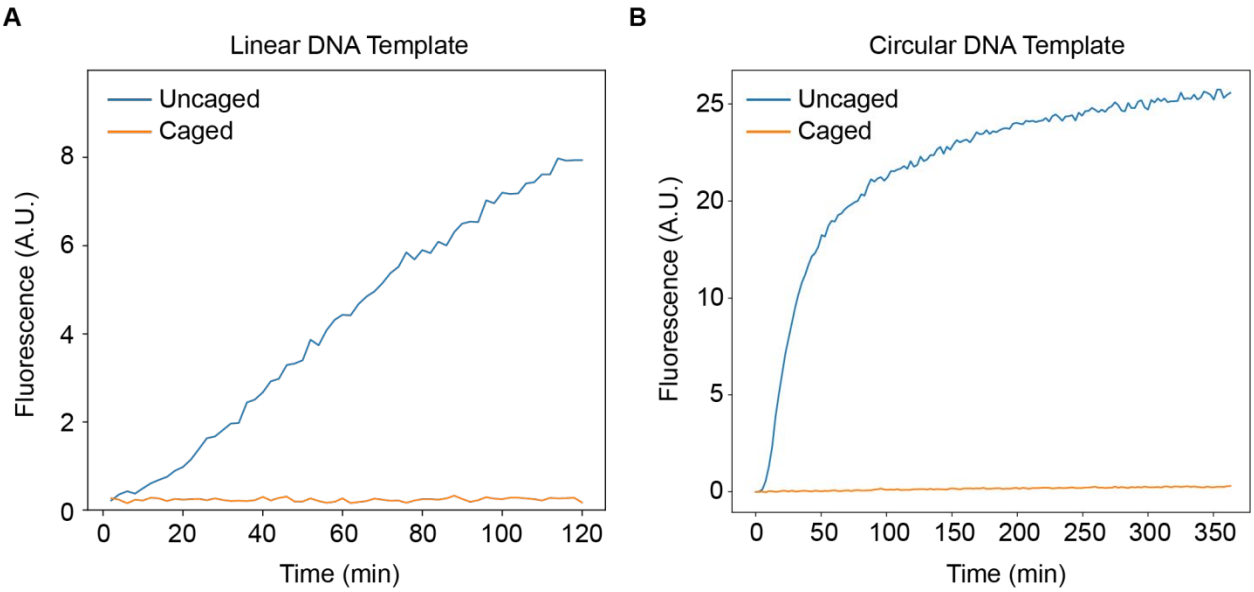


Figure S6. Transcriptional kinetics of caged vs. uncaged RNAP. (A) Run-off transcription using linear DNA template and (B) rolling-circle transcription using circular DNA template. "Caged" (blue curve) refers to the DNA-tagged T7 RNAP in complex with the dsDNA cage molecule. "Uncaged" (orange curve) refers to the DNA template in complex with the T7 RNAP via strand displacement of the dsDNA cage. To uncage the T7 RNAP using the circular DNA template, the latter is catenated to another circular ssDNA called "leash" containing complementary domains to the DNA-tagged T7 RNAP.

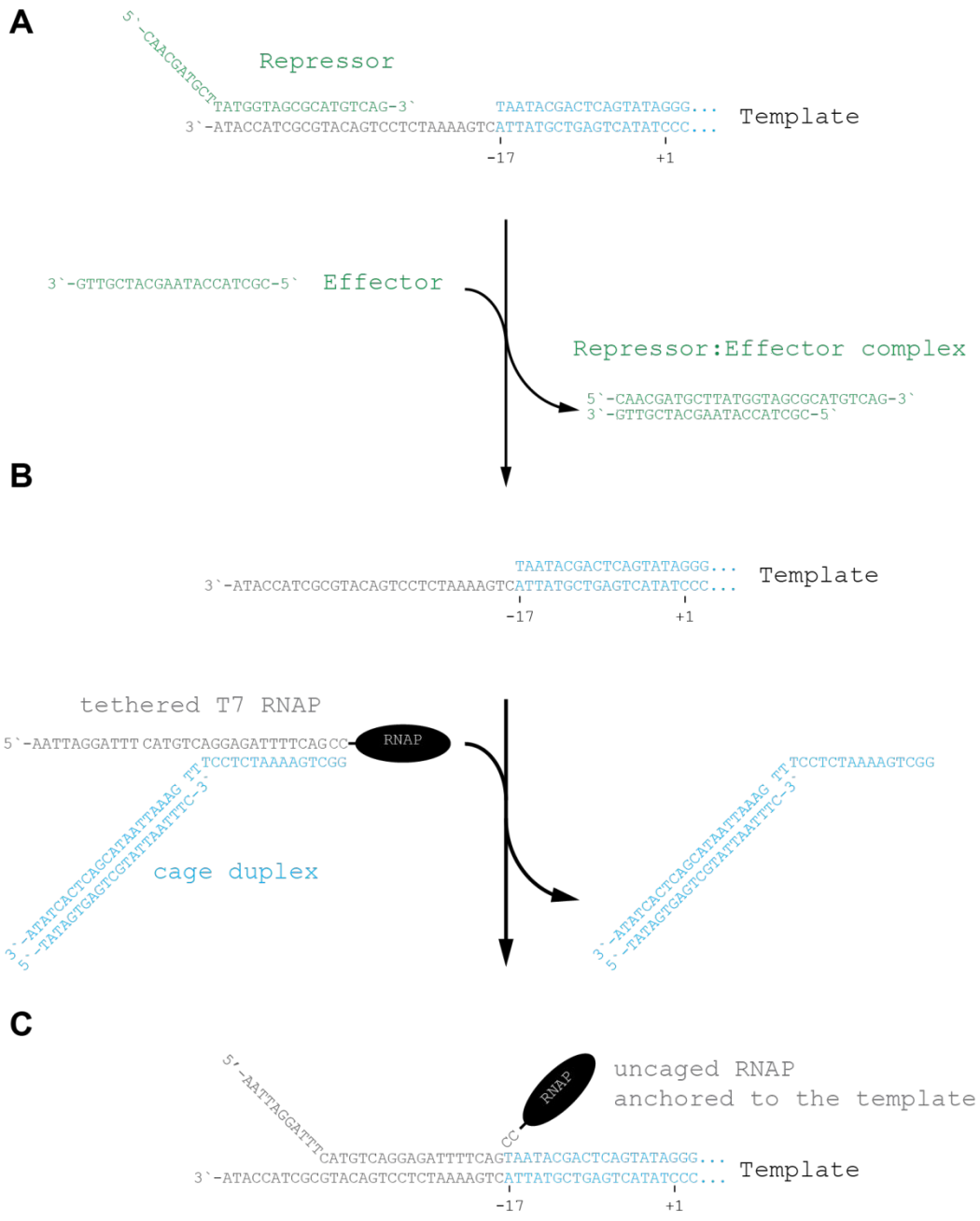


Figure S7. Sequence-level schematic of the *lac* mimic. (A) The initial transcriptionally repressed template, showing just the operator domain (in grey) and promoter region (in light blue) upstream of the gene. A repressor strand (green) binds the operator domain preventing RNAP uncaging. (B) Addition of an effector strand alleviates repression via toehold-mediated strand displacement, liberating the operator domain available for RNAP binding. (C) The transcriptionally active template with the RNAP bound. This occurs via toehold-mediated strand displacement of the cage duplex from the RNAP by the operator domain on the template, leading to anchoring of the uncaged RNAP anchored on the template, which primes the RNAP to initiate transcription of the downstream gene.

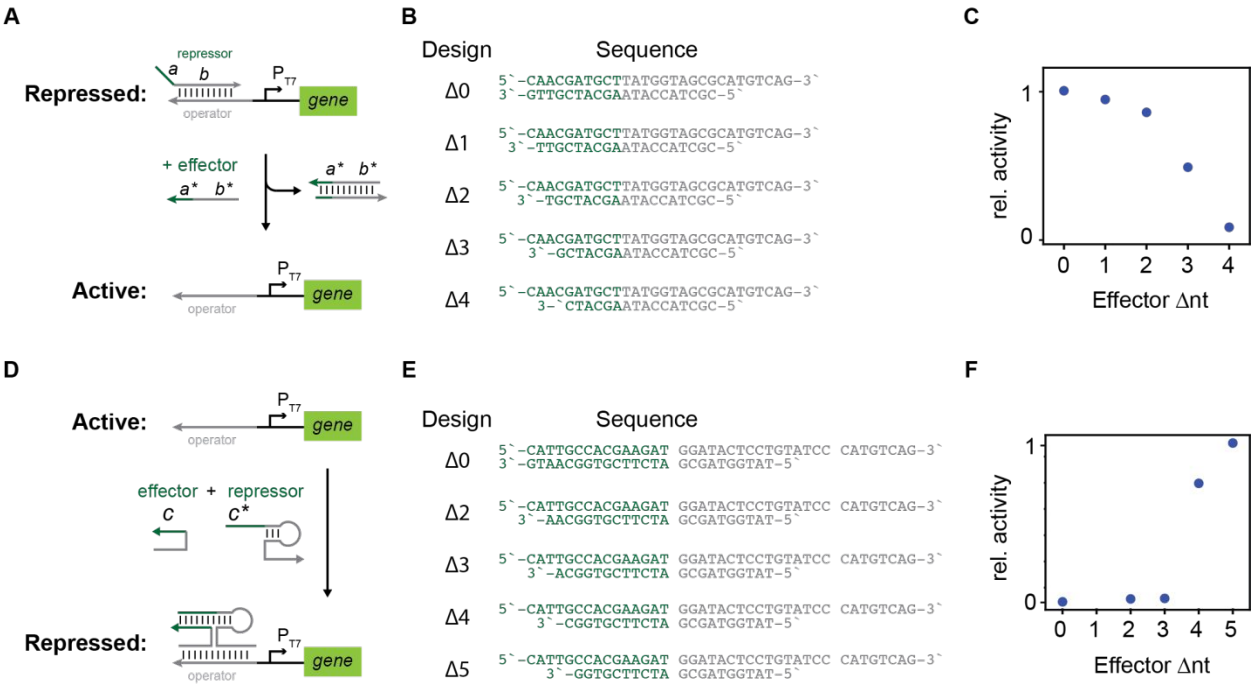


Figure S8. Tunable dose-response of the inducible *lac* and repressible *trp* mimics. (A,D) schematics of the inducible and repressible gene regulatory architecture, respectively. (B,E) Sequences of the effector-repressor complexes for each system, showing progressive 3` nucleotide deletions on the effector strand. (C, F) Dose-response curves of the inducible and repressible systems as a function of the effector sequence designs shown in panels B&E. Response is normalized to the "Δ0" design. For the inducible gene, deletions on the 3` end of the effector reduced its strength of activation; for the repressible gene, deletions on the 3` end of the effector reduced its strength of repression. For C and F, relative activity is normalized to the design with maximum activity (e.g. 1 is maximum activity, and 0 is no activity).

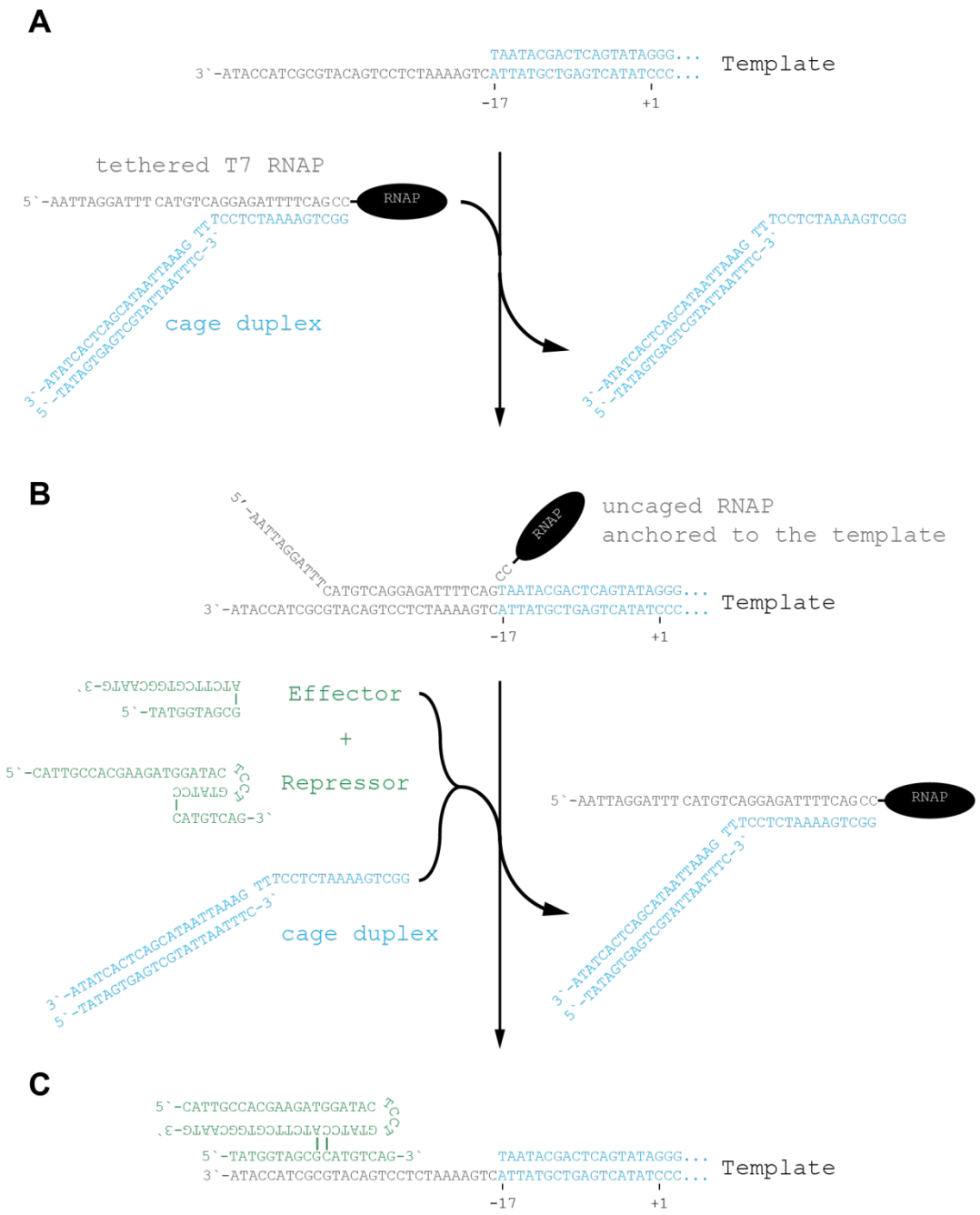


Figure S9. Sequence-level schematic of the *trp* mimic. (A) The initial template, showing just the operator domain (in grey) and promoter region (in light blue) upstream of the gene. (B) Transcriptionally active template bound to an uncaged polymerase. This occurs when the operator domain (in grey) of the template invades the toehold domain on the ssDNA tether of RNAP, releasing its cage duplex into solution and, in the process, anchors the RNAP onto the template. (C) Transcriptionally suppressed template bound to the *trp* effector-repressor complex. This occurs upon the introduction of the effector and repressor strands, which first associate in solution, and forms a four-way junction on the operator domain of the template that partially overlaps with the RNAP binding site. This causes transient dissociation of the RNAP ssDNA tether, which can bind any excess cage duplex in solution to re-cage the RNAP.

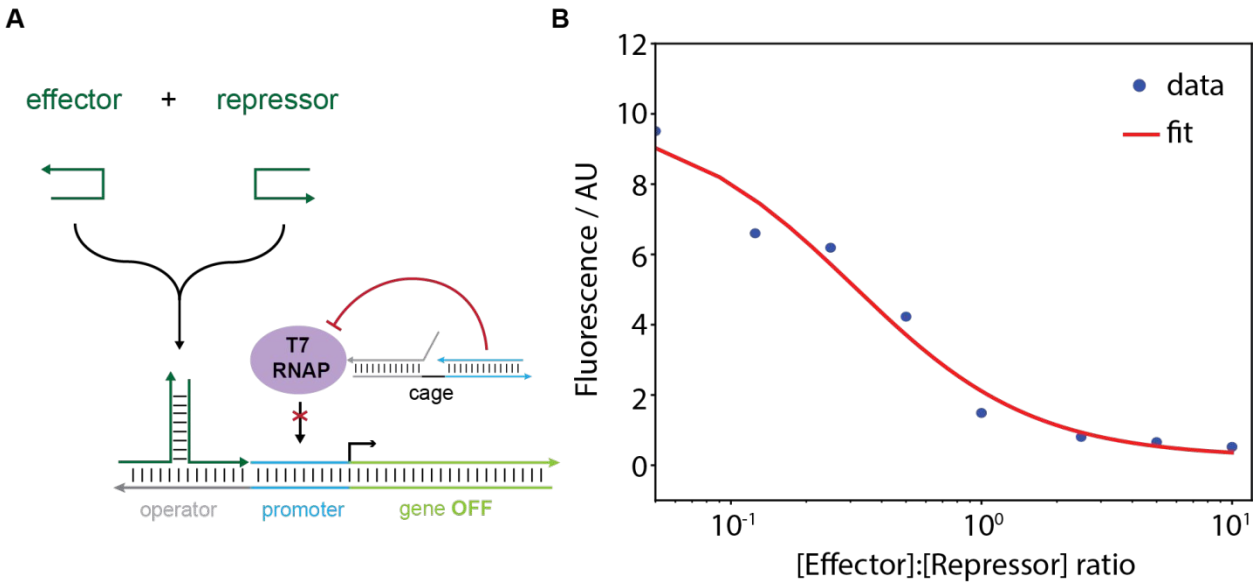


Figure S10. Implementation of repressible gene using three-way-junction (3WJ) motif. We characterized the dose-response of the repressible gene (e.g. the *trp* mimic) in response to a 3WJ repression motif. (A) Schematics of the 3WJ repression motif. (B) Dose-response curve as a function of the effector-repressor stoichiometry. Compared to the four-way junction (4WJ) repressor motif shown in Figure 3E&F, this motif produced a more graded dose-response, e.g. transition from the active to repressed state over a wider range of effector stoichiometry. This is consistent with the weaker binding energetics of 3WJ compared to 4WJ.

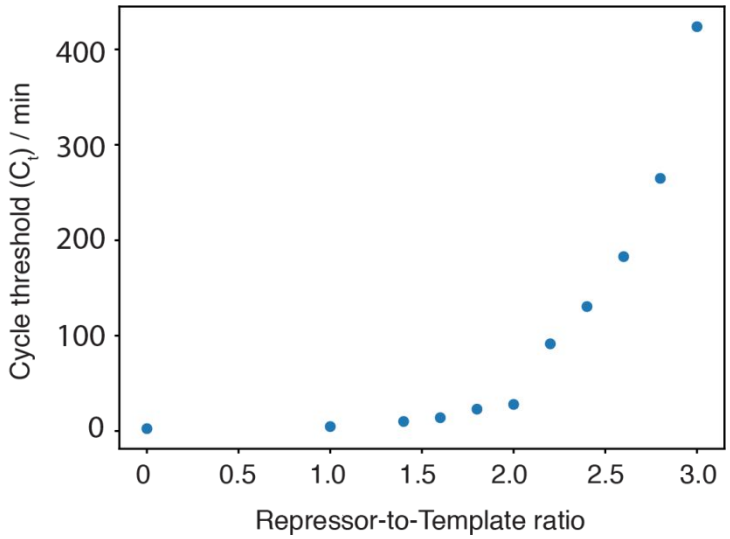


Figure S11. Cycle threshold of autocatalytic template at varying repressor:template ratio. Cycle threshold (C_t) is defined as the time at which the autocatalytic feedback circuit reaches the exponential phase. The value is determined by taking the first derivative of the background subtracted curves shown in Figure 4D, and then determining the inflection point when the slope changes from a zero to a non-zero value. The value for the cycle threshold increases as a function of the repressor-to-template ratio.

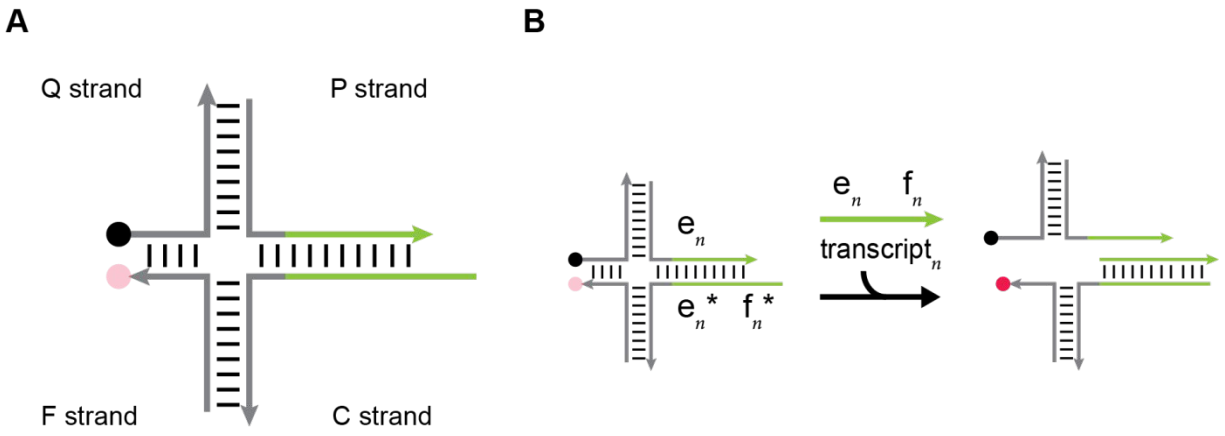


Figure S12. Structure of the molecular beacon. (A) The molecular beacons used in Figure 5 to assay for the identity of the RNA transcripts consist of four strands, named F, Q, P, and C strands. The F strand is labeled on its 3′ end with a fluorophore molecule and the Q strand is labeled on its 5′ end with a quencher molecule. These strands are identical in all the molecular beacons used in this study, whereas the P and C strands are sequence-specific to each RNA transcript (e.g. the light green domain). The C strand, in addition, contains a 7-nt toehold on its 5′ end that allows for strand displacement by an invading nucleic-acid strand. (B) Activation of the molecular beacon by RNA transcripts. The 3′ end of the transcript displaces the Q-P complex via toehold-mediated strand displacement, generating a ternary F-C-transcript complex. The separation between the F and Q strands results in fluorescence recovery.