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DNA-Tethered RNA Polymerase for Programmable In Vitro Transcription and Molecular Computation

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TITLE:

DNA-Tethered RNA Polymerase for Programmable In Vitro Transcription and Molecular Computation

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KEYWORDS:

dynamic DNA nanotechnology, molecular programming, molecular computing, in vitro gene circuit, in vitro transcription, toehold-mediated strand displacement

SUMMARY:

We describe the engineering of a novel DNA-tethered T7 RNA polymerase to regulate in vitro transcription reactions. We discuss the steps for protein synthesis and characterization, validate proof-of-concept transcriptional regulation, and discuss its applications in molecular computing, diagnostics, and molecular information processing.

ABSTRACT:

DNA nanotechnology enables programmable self-assembly of nucleic acids into user-prescribed shapes and dynamics for diverse applications. This work demonstrates that concepts from DNA nanotechnology can be used to program the enzymatic activity of the phage-derived T7 RNA polymerase (RNAP) and build scalable synthetic gene regulatory networks. First, an oligonucleotide-tethered T7 RNAP is engineered via expression of an N-terminally SNAP-tagged RNAP and subsequent chemical coupling of the SNAP-tag with a benzylguanine (BG)-modified oligonucleotide. Next, nucleic-acid strand displacement is used to program polymerase transcription on-demand. In addition, auxiliary nucleic acid assemblies can be used as "artificial transcription factors" to regulate the interactions between the DNA-programmed T7 RNAP with its DNA templates. This in vitro transcription regulatory mechanism can implement a variety of circuit behaviors such as digital logic, feedback, cascading, and multiplexing. The scalability and composability of this gene regulatory architecture facilitates design abstraction, standardization, and scaling and hence, rapid prototyping of in vitro genetic devices for applications such as bio-sensing, disease detection, and data storage.

INTRODUCTION:

DNA computing uses a set of designed oligonucleotides as the medium for computation. These oligonucleotides are programmed with sequences to dynamically assemble according to user-

specified logic and respond to specific nucleic-acid inputs. In proof-of-concept studies, the output of the computation typically consists of a set of fluorescently labelled oligonucleotides that can be detected via gel electrophoresis or fluorescence plate readers. Over the past 30 years, increasingly complex DNA computational circuitries have been demonstrated, such as various digital logic cascades, chemical reaction networks, and neural networks[1–3]. To assist with the preparation of these DNA circuits, mathematical models have been used to predict the functionality of synthetic gene circuits[4,5], and computational tools have been developed for orthogonal DNA sequence design[6–10]. Compared to silicon-based computers, the advantages of DNA computers include their ability to interface directly with biomolecules, operate in solution in the absence of a power supply, as well as their overall compactness and stability. With the advent of next-generation sequencing, the cost of synthesizing DNA computers has been decreasing for the past two decades at a rate faster than Moore's Law[11]. Applications of such DNA-based computers are now beginning to emerge, such as for disease diagnosis[12,13], for powering molecular biophysics[14], and as data storage platforms[15].

[Insert Figure 1 here]

To-date, the majority of DNA computers utilize a well-established motif in the field of dynamic DNA nanotechnology known as toehold-mediated DNA strand displacement (TMDSD, **Figure 1**)[16]. This motif consists of a partially double-stranded DNA (dsDNA) duplex displaying short "toehold" overhangs (i.e., 7- to 10 nucleotides (nt)). Nucleic acid "input" strands can interact with the partial duplexes through the toehold. This leads to the displacement of one of the strands from the partial duplex, and this liberated strand can then serve as input for downstream partial duplexes. Thus, TMDSD enables signal cascading and information processing. In principle, orthogonal TMDSD motifs can operate independently in solution, enabling parallel information processing. There have been a number of variations on the TMDSD reaction, such as toehold-mediated DNA strand exchange (TMDSE)[17], "leakless" toeholds with double-long domains[18], sequence-mismatched toeholds[19], and "handhold"-mediated strand displacement[20]. These innovative design principles allow more finely tuned TMDSD energetics and dynamics for improving DNA computing performance.

Synthetic gene circuits, such as transcriptional gene circuits, are also capable of computation[21–23]. These circuits are regulated by protein transcription factors, which activate or repress transcription of a gene by binding to specific regulatory DNA elements. Compared to DNA-based circuits, transcriptional circuits have several advantages. First, enzymatic transcription has a much higher turnover rate than existing catalytic DNA circuits, thus generating more copies of output per single copy of input and providing a more efficient means of signal amplification. In addition, transcriptional circuits can produce different functional molecules, such as aptamers or messenger RNA (mRNA) encoding for therapeutic proteins, as computation outputs, which can be exploited for different applications. However, a major limitation of current transcriptional circuits is their lack of scalability. This is because there is a very limited set of orthogonal protein-based transcription factors, and de novo design of new protein transcription factors remains technically challenging and time-consuming.

This paper introduces a novel building block for molecular computing that combines the functionalities of transcriptional circuits with the scalability of DNA-based circuits. This building block is a T7 RNAP covalently attached with a single-stranded DNA tether (**Figure 2A**). To synthesize this DNA-tethered T7 RNAP, the polymerase was fused to an N-terminal SNAP-tag[24] and recombinantly expressed in *Escherichia coli*. The SNAP-tag was then reacted with an oligonucleotide functionalized with the BG substrate. The oligonucleotide tether allows the positioning of molecular guests in close proximity to the polymerase via DNA hybridization. One such guest was a competitive transcriptional blocker referred to as a "cage", which consists of a "faux" T7 promoter DNA duplex with no gene downstream (**Figure 2B**). When bound to the RNAP via its oligonucleotide tether, the cage stalls polymerase activity by outcompeting other DNA templates for RNAP binding, rendering the RNAP in an "OFF" state (**Figure 2C**).

To activate the polymerase to an "ON" state, T7 DNA templates with single-stranded "operator" domains upstream of the T7 promoter of the gene were designed. The operator domain (i.e., domain *a*b** **Figure 2C**) can be designed to displace the cage from the RNAP via TMDSD and position the RNAP proximal to the T7 promoter of the gene, thus initiating transcription. Alternatively, DNA templates were also designed where the operator sequence was complementary to auxiliary nucleic-acid strands that are referred to as "artificial transcription factors" (i.e., TF_A and TF_B strands in **Figure 3A**). When both strands are introduced into the reaction, they will assemble at the operator site, creating a new pseudo-contiguous domain *a*b**. This domain can then displace the cage via TMDSD to initiate transcription (**Figure 3B**). These strands can be supplied either exogenously or produced.

[Insert Figure 3 here]

The use of nucleic acid-based transcription factors for in vitro transcriptional regulation allows the scalable implementation of sophisticated circuit behaviors such as digital logic, feedback, and signal cascading. For example, one can build logic gate cascades by designing nucleic acid sequences such that the transcripts from an upstream gene activate a downstream gene. One application that exploits the cascading and multiplexing made capable by this proposed technology is the development of more sophisticated molecular computing circuitries for portable diagnostics and molecular data processing. In addition, integrating the molecular computing and de novo RNA synthesis capabilities can enable new applications. For example, a molecular circuit can be designed to detect one or a combination of user-defined RNAs as inputs and output therapeutic RNAs or mRNAs encoding functional peptides or proteins for point-of-care medical applications.

PROTOCOL:

1. Buffer preparation

NOTE: Protein purification buffer preparation can occur on any day; here, it was done prior to beginning the experiments.

1.1. Prepare lysis/equilibration buffer containing 50 mM tris(hydroxymethyl)aminomethane (Tris), 300 mM sodium chloride (NaCl), 5% glycerol, and 5 mM β-mercaptoethanol (BME), pH 8. Add 1.5 mL of 1M Tris, 1.8 mL of 5M NaCl, 1.5 mL of glycerol, 25.2 mL of deionized water (ddH_2O) into a 50 mL centrifuge tube, and add 10.5 µL of 14.2 M BME just prior to use.

NOTE: Tris can cause acute toxicity; hence, avoid breathing its dust, and avoid skin and eye contact. BME is toxic and should only be used in a fume hood. It is important to add BME last, just prior to resuspension and cell lysis. See **Table 1** for lysis buffer formula.

1.2. Prepare wash buffer (pH 8) containing 50 mM Tris, 800 mM NaCl, 5% glycerol, 5 mM BME, and 20 mM imidazole. Add 1.5 mL of 1 M Tris, 4.8 mL of 5 M NaCl, 1.5 mL of glycerol, and 22.2 mL of ddH_2O into a 50 mL centrifuge tube. Just prior to use, add 7 µL of 14.2 M BME and 200 µL of 2 M imidazole to 20 mL of the above solution.

NOTE: To prevent acute toxicity due to imidazole, use personal protective equipment. It is important to add BME and imidazole last, just prior to washing the protein out of the column. See **Table 2** for wash buffer formula.

1.3. Prepare elution buffer (pH8) containing 50 mM Tris, 800 mM NaCl, 5% glycerol, 5 mM BME, and 200 mM imidazole. Add 0.5 mL of 1 M Tris, 1.6 mL of 5 M NaCl, 0.5 mL of glycerol, and 6.4 mL of ddH_2O to a 15 mL centrifuge tube. Just prior to use, add 3.5 µL of 14.2 M BME and 1 mL of 2 M imidazole to 10 mL of the above solution.

NOTE: It is important to add BME and imidazole last, just prior to eluting the protein out of the column. See **Table 3** for elution buffer formula.

1.4. Prepare 2x storage buffer (to be mixed 1:1 with glycerol) containing 100 mM Tris, 200 mM NaCl, 40 mM BME, and 2 mM ethylenediaminetetraacetic acid (EDTA), 0.2% of a non-ionic surfactant (see the **Table of Materials**). Prepare 50 mL of the storage buffer by adding 5 mL of 1 M Tris, 2 mL of 5 M NaCl, 42.56 mL of ddH_2O, 200 µL of 0.5 M EDTA, 100 µL of the non-ionic surfactant to a 50 mL centrifuge tube. Mix until the solution is homogeneous, filter the storage buffer through a 0.2 µm syringe filter, and add 140.8 µL of BME to the above solution prior to use.

NOTE: To avoid acute toxicity due to EDTA, avoid breathing its dust, and avoid skin and eye contact. It is important to add BME last and mix the entire storage buffer 1:1 with glycerol, just prior to storing the purified protein. See **Table 4** for storage buffer formula.

2. Overnight culture growth: Day 1

2.1. Prepare 1,000x kanamycin stock by dissolving 500 mg of kanamycin in 10 mL of ddH_2O.

NOTE: Use personal protective equipment to prevent acute toxicity due to kanamycin.

2.2. Add 20 µL of the 1,000x kanamycin stock to 20 mL of lysogeny broth. Using a sterile pipette tip, poke a transformed BL21 *E. coli* glycerol stock and then inoculate the culture by introducing the tip into the growth media broth.

[Insert Figure 4 here]

NOTE: The plasmid encodes a T7 RNAP containing an N-terminal histidine tag and a SNAP-tag domain (SNAP T7 RNAP), as well as a kanamycin resistance gene under a pQE-80L backbone (**Figure 4**).[25]

2.3. Again, add 20 µL of the 1,000x kanamycin stock to a separate culture flask containing 20 mL of lysogeny broth, and incubate it as a control.

2.4. Incubate the two samples (from steps 2.2 and 2.3) overnight for 12–18 h at 37 °C, while rotating at 10 × *g*.

3. Cell growth and induction: Day 2

3.1. Inoculate 400 mL of lysogeny broth containing 400 µL of kanamycin stock with 4 mL of the overnight growth culture from step 2.4. Incubate the culture flasks at 37 °C, while rotating at 10 × *g*.

3.2. Once the culture has reached an optical density (OD) at 600 nm of ~0.5, take out 1 mL of sample from the growth flask as a control. Store the control sample at 4 °C.

3.3. Induce the cells with isopropyl β-D-1-thiogalactopyranoside (IPTG) by adding 40 µL of 1M IPTG per 100 mL of culture to achieve a final concentration of 0.4 mM IPTG. Incubate the sample for 3 h at 37 °C, rotating at 10 × *g*, and then spin the induced culture at 8,000 × *g* for 10 min to pellet the cells. Remove the supernatant, and store the pellet at –20 °C until further use.

NOTE: To avoid acute toxicity due to IPTG, avoid breathing its dust, and avoid skin and eye contact. If necessary, you can pause the experiment here and continue the next day.

4. Cell lysis, protein purification: Day 3

4.1. Resuspend the stored cell pellet with 10 mL of lysis buffer on ice, and gently swirl to ensure the entire pellet is resuspended. Then, pipette 1 mL of sample into ten 1.5 mL tubes that are kept on ice.

4.2. Sonicate each sample at an amplitude setting of “1”, pulsed for 2 s with a 50% duty cycle over a period of 30 s. Before and after each sample, clean the sonication tip with 70% ethanol and ddH_2O. Keep all samples on ice during and after sonication.

NOTE: Keep 70% ethanol away from heat and open flame.

4.3. Equilibrate a nickel-charged nitrilotriacetic acid (Ni-NTA) purification spin column to a working temperature of 4 °C. Place/store the column at 4 °C, and keep on ice during use.

4.4. Centrifuge the ten 1 mL samples at 15,000 × *g* for 20 min at 4 °C. Carefully pipette out the supernatant containing the recombinant RNAP without disturbing the pellet. If necessary, use additional equilibration buffer to adjust the total volume to ≥ 6 mL.

4.5. Gently remove the bottom tab from the Ni-NTA spin column to allow for flow through the column. Place the column in a centrifuge tube, and keep it on ice.

NOTE: Use a 50 mL centrifuge tube with the 3 mL Ni-NTA spin columns.

4.6. Centrifuge the column at 700 × *g* and 4 °C for 2 min to remove the storage buffer. Equilibrate the column by adding 6 mL of equilibration buffer to the column. Allow the buffer to fully enter the resin bed.

4.7. Remove the equilibration buffer from the column by centrifugation at 700 × *g* and 4 °C for 2 min. Before adding the prepared cell extract to the column, place a bottom plug on the column to avoid losing any product. Then, add the cell extract to the column, and mix on an orbital shaker mixer for 30 min at 4 °C.

4.8. Remove the bottom plug from the column and place the column in a 50 mL centrifuge tube labeled **flow through**. Centrifuge the column at 700 × *g* for 2 min to collect the flow through.

4.9. Add 6 mL of wash buffer to the column to wash the resin. Centrifuge the column at 700 × *g* for 2 min to collect the fraction in a new centrifuge tube labeled **wash 1.** Repeat this step two more times for a total of 3 separate fractions, and collect the fractions in separate centrifuge tubes (**wash 2** and **wash 3**).

4.10. Add 3 mL of elution buffer to elute the His-tagged proteins from the resin. Centrifuge the column at 700 × *g* for 2 min to collect fraction in a new centrifuge tube labeled **eluate 1**. Repeat this step two more times for a total of 3 separate fractions, and collect the fractions into separate centrifuge tubes (**eluate 2** and **eluate 3**).

4.11. Combine the eluates and perform desalting to remove salts from the protein solution.

4.11.1. Pipette 15 mL of 0.05 % w/v polysorbate 20 over a 100 kDa centrifugal filter unit. Centrifuge at 4,000 × *g* for 40 min and discard the flow-through.

4.11.2. Use the coated filter to concentrate the eluates 1, 2, and 3 (9 mL of total of protein eluate + 6 mL of storage buffer) to ~1,500 µL. Centrifuge the filter at 3,220 × *g* for 20 min, and gently pipette-wash the membrane to prevent precipitation.

4.11.3. Dilute the sample to 15 mL with storage buffer. Perform a buffer exchange using storage buffer 1:1,000 by repeating step 4.11.2 two more times.

4.12. Quantify the purified protein by measuring the absorbance of the fraction at 280 nm. Blank the spectrophotometer with storage buffer (2x storage buffer at 4 °C). Gently mix the sample of the combined eluates and measure its absorbance.

NOTE: Perform three separate readings at 1x, 10x, and 50x dilutions of the protein sample to average and quantify the protein. Dilute samples in storage buffer.

4.13. Adjust the protein samples to 100 µM using 2x storage buffer. Dilute the adjusted sample 1:1 by volume with 100% glycerol. Store the resulting protein solution at -80 °C.

5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein product: Day 3

5.1. Run an SDS-PAGE gel for protein analysis. Mix 9 µL of the sample with 3 µL of 4x lithium dodecyl sulfate (LDS) protein loading dye. Heat the samples at 95 °C for 10 min.

5.2. Load the samples onto a 4–12% Bis-Tris SDS-PAGE gel setup. Load the protein ladder in well 1, then with samples (from left to right): flow-through, wash 1, wash 2, wash 3, elution 1, elution 2, elution 3, and total desalted elution.

NOTE: **Table 5** contains a sample loading table for the SDS-PAGE gel.

5.3. Run the loaded gel samples in 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer for 35 min at 200 V. Rinse the gel in a clean tray three times for 10 min each using 200 mL of ddH_2O, with gentle agitation to remove any SDS from the gel matrix.

NOTE: Wear personal protective equipment to avoid acute toxicity due to the MES.

5.4. Stain the gel with 20 mL of Coomassie blue, and incubate the gel overnight at room temperature with gentle agitation. De-stain the gel twice for 1 h each with 200 mL of ddH_2O with gentle agitation on an orbital shaker.

NOTE: Washing the gel for a longer period or frequently replacing the water will enhance sensitivity. Additionally, placing a folded delicate-task wipe tissue in the container to absorb excess dye will accelerate the de-staining process.

6. Functional verification of SNAP T7 RNAP via in vitro transcription

NOTE: This protocol uses DNA template, which encodes for the fluorescent Broccoli RNA aptamer and allows the use of fluorescence to monitor the kinetics of transcription on a fluorescence plate reader.

6.1. Set up three in vitro transcription (IVT) reactions to compare the activity of SNAP T7 RNAP with wild-type (WT) T7 RNAP from a commercial source and a buffer-only control. Adjust the volume of each reaction to 20 µL.

6.1.1. Prepare the SNAP T7 RNAP IVT reaction by mixing 2 µL of 10x transcription buffer, 0.4 µL of 25 mM ribonucleoside triphosphate (rNTP) mix, 5 µL of 500 nM DNA template, 2 µL of 500 nM SNAP T7 RNAP, and 10.6 µL of ddH_2O.

6.1.2. Prepare the WT RNAP IVT reaction by mixing 2 µL of 10x transcription buffer, 0.4 µL of 25 mM rNTP mix, 5 µL of 500 nM DNA template, 2 µL of WT T7 RNAP, and 10.6 µL of ddH_2O.

6.1.3. Prepare the buffer-only IVT reaction by mixing 2 µL of 10x transcription buffer, 0.4 µL of 25 mM rNTP mix, 5 µL of 500 nM DNA template, and 12.6 µL of ddH_2O.

NOTE: Add the RNAP last, keeping the samples on ice until its introduction. **Table 6**, **Table 7**, and **Table 8** contain the IVT reaction formulas.

6.2. Monitor the transcription kinetics on a fluorescence plate reader for 2 h at 2 min intervals at 37 °C using an excitation wavelength of 470 nm and an emission wavelength of 512 nm.

7. Preparation of BG-modified oligonucleotides: Day 1

7.1. Dissolve the oligonucleotide with 3'-amine modification in ddH_2O to a final concentration of 1 mM. Label this **S1**.

7.1.1. Mix 25 µL of 1 M sodium bicarbonate ($NaHCO_3$), 284 µL of 100% dimethyl sulfoxide (DMSO), 125 µL of S1 (oligonucleotide stock), and 66 µL of 50 mM of the BG-*N*-hydroxysuccinimide (NHS) ester (BG-GLA-NHS) diluted with DMSO, adjust the volume to 500 µL, and incubate overnight at room temperature at 100 × *g*.

NOTE: Keep DMSO away from heat and flame as it is a combustible liquid. **Table 9** contains the reaction formula for the BG conjugation to the oligonucleotide.

8. Ethanol/acetone precipitation of BG-oligonucleotide conjugate: Day 2

8.1. Centrifuge the product of step 7.1.1. at 13,000 × *g* for 5 min. Carefully transfer the supernatant to a fresh tube and discard any precipitated BG. Split the reaction into two equal 250 µL aliquots to prevent overflow, and perform the following steps on both aliquots.

8.2. Add $1/10^{th}$ of the volume of 3 M sodium acetate (25 µL), followed by 2.5x the volume in 100% ethanol (625 µL). Incubate at –80 °C for 1 h.

NOTE: Use personal protective equipment when handling both sodium acetate (may cause irritation to eyes, skin, digestive and respiratory tract) and ethanol (extremely flammable, causes irritation on contact). If necessary, pause the experiment here and continue the next day.

8.3. Place the tubes in the centrifuge, and mark the outer edge. Centrifuge the tubes at 17,000 × *g* for 30 min at 4 °C.

NOTE: The oligonucleotide pellet will appear on the marked edge of the tube.

8.4. Without disturbing the pellet, discard the supernatant. Top up with 750 µL of chilled 70% ethanol, and spin at 17,000 × *g* for 10 min at 4 °C.

8.5. Without disturbing the pellet, discard the supernatant. Top up with 750 µL of 100% acetone, and spin at 17,000 × *g* for 10 min at 4 °C.

NOTE: Use personal protective equipment when handling acetone as it is extremely flammable and causes irritation on contact.

8.6. With the tube lid open, air dry for 5 min to remove any excess acetone through evaporation. Re-dissolve the oligonucleotide in 250 µL of 1x Tris-EDTA (TE) buffer to produce a ~850 µM BG-oligonucleotide solution.

8.7. Repeat steps 8.2 to 8.6, and re-dissolve in 70 µL of 1x TE buffer. Label this **S2**.

9. BG-oligonucleotide cleanup via gel filtration chromatography

9.1. Suspend the matrix by vigorously inverting the columns several times; remove the top cap and snap off the bottom tip of column. Place the column in a 1.5 mL centrifuge tube, and centrifuge the tube at 1,000 × *g* for 1 min at room temperature. Discard the eluted buffer and collection tube.

NOTE: It is important to prevent vacuum formation. Use prepared columns immediately.

9.2. Place the packed columns in clean 1.5 mL centrifuge tubes. Add 300 µL of 1x TE buffer to the center of the column bed, and centrifuge at 1,000 × *g* for 2 min to exchange the buffer solution. Once again, discard the eluted buffer and collection tube.

9.3. Place the buffer-exchanged columns in clean 1.5 mL centrifuge tubes. Apply up to 75 µL of sample to the center of the bed. Spin at 1,000 × *g* for 4 min.

NOTE: Do not disturb the bed or touch the sides of the column; the highest point of the gel media should point toward the outside rotor.

9.4. Collect the eluate from the collection tube, as it contains the purified nucleic acid. To quantify the sample, measure its absorbance at 260 nm; label this **S3**.

NOTE: Note the path length used in the measurement, and calculate the concentration using the Beer-Lambert law.

10. Denaturing PAGE analysis of BG-oligonucleotide conjugate

10.1. Cast a 18% Tris-borate-EDTA (TBE)-Urea PAGE gel. Dissolve 4.8 g of UREA, 4.5 mL of 40% acrylamide (19:1), and 1 mL of 10x TBE in 2.8 mL of ddH_2O; add 5 µL tetramethylethylenediamine (TEMED) and mix thoroughly. Repeat with 100 µL of 10% ammonium persulfate (APS). Pour the solution into an empty gel cassette and allow polymerization for 40 min.

NOTE: Use appropriate personal protective equipment when handling urea (causes irritation to eyes and skin), acrylamide (toxic and carcinogenic), and TEMED (toxic, flammable, corrosive). **Table 10** contains the reaction formula for an 18% TBE-UREA polyacrylamide gel.

10.2. Microwave 500 mL of TBE buffer (0.5x) for 2 min and 30 s or until ~70 °C and pour into a gel apparatus. Prepare formamide (denaturing) loading dye containing 95% formamide + 1 mM EDTA and bromophenol blue. Mix the loading dye with each sample, and load the mixture onto the polyacrylamide gel.

Note: Use appropriate personal protective equipment when handling formamide as it is carcinogenic. **Table 11** contains a sample gel loading table.

10.3. Run the gel at 270 V for 35 min, or until the dye front migrates to the end. Place the gel in a gel box and stain with cyanine dye for nucleic acids for 15 min at room temperature before imaging.

NOTE: Use appropriate personal protective equipment when handling cyanine dye as it is combustible.

11. Conjugation of oligonucleotide to SNAP T7 RNAP and PAGE analysis

11.1. Prepare the reagents for the analytical-scale coupling of BG-oligonucleotide to SNAP T7 RNAP: make 9 dilutions of single-stranded DNA (ssDNA) oligo with ddH_2O to create oligo:RNAP ratios ranging from 5:1 to 1:5. Dilute the protein stock to 50 µM.

NOTE: Example ratios can be found in **Table 12**; these ratios are calculated using a RNAP concentration of 50 µM.

11.2. For each dilution of ssDNA oligo, make 10 µL of the reaction mixture containing 2 µL of SNAP buffer, 4 µL of BG-oligonucleotide, and 4 µL of SNAP T7 RNAP.

NOTE: **Table 13** contains reaction formulas for the SNAP-tag labeling reaction.

11.2.1. Prepare two more control samples: 1) an RNAP control by replacing BG-oligonucleotide with ddH_2O; 2) a DNA control by replacing SNAP T7 RNAP with ddH_2O (for the lowest oligonucleotide concentration of SNAP T7 RNAP). Incubate all samples at room temperature for 1 h, and keep on ice until needed.

11.3. Set up eleven 10 µL reactions by adding 2 µL of each sample to 4 µL of SNAP buffer and 2 µL of protein loading dye, and heat at 70 °C for 10 min. Load 2 µL of each sample onto the 4–12% Bis-Tris protein gel, and perform gel electrophoresis on ice at 200 V for 35 min.

NOTE: **Table 14** contains reaction formulas for the gel loading samples.

11.3.1. Wash SDS off via 3x water-exchange on a shaker, each wash lasting 10 min each. Stain with cyanine dye for nucleic acids for 15 min before imaging. Stain the gel again using 20 mL of Coomassie blue stain for 1 h. De-stain with ddH_2O for 1 h (or overnight) before imaging.

NOTE: In the gel, one of the reactions will produce the most tethered polymerase along with the least amount of excess free BG-oligonucleotide; this is the optimal ratio.

11.4. Prepare reagents for the preparative scale coupling BG-oligonucleotide to SNAP T7 RNAP. Perform the coupling reaction with the optimal ratio found in the analytical scale.

NOTE: Minimize protein exposure to room temperature by placing the protein on ice when not in use.

12. Purification of oligonucleotide-tethered SNAP-T7 using ion exchange columns

12.1. Follow the manufacturer's instructions for tube setup if it deviates from the instructions listed here. Prepare a purification buffer with pH higher than the isoelectric point of the protein.

NOTE: For the example protein in this protocol, a purification buffer of 10 mM sodium phosphate buffer (pH 7) was used.

12.1.1. Prepare 1,000 µL of elution buffer containing final concentrations of 50 mM Tris and 0.5 M NaCl. Mix 50 µL of 1 M Tris, 100 µL of 5 M NaCl, and 850 µL of ddH_2O.

NOTE: **Table 15** contains the reaction formula for the elution buffer.

12.2. Place a column in a 2 mL centrifuge tube, and wash with purification buffer at 2,000 × *g* for 15 min, or until all the buffer has been eluted. Discard the eluted buffer.

12.3. Dilute each sample with purification buffer at a 3:1 purification buffer:sample ratio, and load the sample into the column 400 µL at a time. Spin at 2,000 × *g* for 10 min, or until all the buffer has been eluted. Collect the flow-through and label it as **flow-through**.

12.4. Add 400 µL of purification buffer into the center of the column. Spin at 2,000 × *g* for 15 min, or until all the buffer has been eluted. Collect the flow-through and label it as **wash 1**. Repeat twice more for **wash 2** and **wash 3**.

12.5. Add 50 µL of elution buffer into the center of the column. Spin at 2,000 × *g* for 5 min, or until all the buffer has been eluted. Collect the flow-through and label it as **eluate 1**. Repeat twice more for **eluate 2** and **eluate 3**.

12.6. Pool eluates 1, 2, and 3 (label this **total eluate**), leaving a small fraction of each eluate for the gel, and measure absorbance at 260 nm (A260) and 280 nm (A280). After the measurement, add glycerol at a 1:1 ratio and store at –20 °C until further use.

12.7. Use a centrifugal filter unit (0.5 mL; 30 kDa) to buffer-exchange **total eluate** with 2x storage buffer (~1:100) (label this **product**). Measure A260/280 again. Add glycerol at a 1:1 ratio and store at –20 °C until further use.

12.8. Load each eluate: flow-through, wash 1–3, total eluate, and product in a 4–12% Bis-Tris SDS-PAGE gel, along with a protein ladder. Run at 200 V for 35 min, or until the dye front migrates to the end.

13. Demonstration of on-demand control of tethered RNA polymerase activity

13.1. Prepare 5x annealing buffer containing 25 mM Tris, 5 mM EDTA, and 25 mM magnesium chloride ($MgCl_2$). Mix 2.4 µL of each template (1 µM) with 5 µL of annealing buffer and 14.2 µL of ddH_2O to form 25 µL of 1 µM dsDNA cage. Incubate this solution at 75 °C for 2 min. Similarly, anneal the sense and antisense strands of the promoter and malachite green aptamer DNA template. Prepare a 1mM solution of malachite green oxalate.

NOTE: **Table 16** contains the reaction formula for 5x annealing buffer, **Table 17** contains the reaction formula for annealing two ssDNA templates.

13.2. Incubate the tethered SNAP T7 RNAP with the dsDNA cage in a 1:5 molar ratio at room temperature for 15 min to a final concentration of 500 nM RNAP. Keep on ice until needed.

13.3. Preheat the plate reader to 37 °C. Set up three 25 µL IVT reactions on ice.

13.3.1. Set up a reaction containing the caged SNAP T7RNAP with nucleic acid transcription factors. Mix 2.5 µL of 10x IVT buffer, 1 µL of 25 mM rNTP mix, 1 µL of 1 mM malachite green, 2.5 µL of the RNAP-cage mixture, 2.5 µL each of 1 µM transcription factor A and B oligonucleotide strands, and 3 µL of 1 mM malachite green aptamer template in 10 µL of ddH_2O.

13.3.2. Set up a reaction containing the caged SNAP T7RNAP without nucleic acid transcription factors. Mix 2.5 µL of 10x IVT buffer, 1 µL of 25 mM rNTP mix, 1 µL of 1 mM malachite green, 2.5 µL of the RNAP-cage mixture, and 3 µL of 1 mM malachite green aptamer template in 15 µL of ddH_2O.

13.3.3. Set up a reaction containing buffer only. Mix 2.5 µL of 10x IVT buffer, 1 µL of 25 mM rNTP mix, 1 µL of 1 mM malachite green, and 3 µL of 1 mM malachite green aptamer template in 17.5 µL of ddH_2O.

NOTE: **Table 18** contains a general reference for the in vitro transcription reactions.

13.4. Transfer each reaction to a 384-well plate. Monitor transcription of the malachite green aptamer on a fluorescence plate reader for 2 h at 37 °C and with 610 nm excitation and 655 nm emission. Once finished, keep the plate on ice until needed.

13.5. Microwave 0.5x TBE buffer for 2 min 30 s or until ~70 °C. Run the RNA products of each well in a denaturing 12% TBE-Urea polyacrylamide gel in the heated 0.5x TBE buffer at 280 V for 20 min, or until the dye front reaches the end. Stain the gel with cyanine dye nucleic acid stain for 10 min on an orbital shaker before imaging.

NOTE: **Table 19** contains the reaction formula for a denaturing 12% TBE-Urea PAGE gel.

REPRESENTATIVE RESULTS:

[Insert Figure 5 here]

Successful expression and purification of the recombinant SNAP T7 RNAP protein was confirmed using SDS-PAGE (**Figure 5A**). The band for SNAP T7 RNAP is expected at approximately 119 kDa, consistent with the molecular weights of the wild-type T7 RNAP and the SNAP-tag being 99 kDa and 20 kDa, respectively. The His-tag purification procedure described here produced a total of seven fractions, consisting of a flow-through fraction (FT), three wash fractions (W1, W2, and W3), and three elution fractions (E1, E2, and E3). In addition, an aliquot of the protein following buffer exchange and up-concentration (DE) is also typically included. As seen in **Figure 5A**, the most prominent band appeared at approximately 119 kDa in the elution fractions, suggesting successful protein expression. The majority of the flow-through and wash fractions contained crude cell lysates. A minor portion of the cell lysates carried over to the elution fractions, suggesting that more stringent washes needed to be performed, although this may reduce yield of the protein product. In addition to the main SNAP T7 RNAP band, a second, less prominent band was observed at approximately 20 kDa, which was attributed to truncated SNAP-tag protein. Based on band intensity, this by-product was significantly lower in concentration compared to the SNAP T7 RNAP. It can be removed by an additional round of size-exclusion chromatography or diafiltration with 100 kDa molecular weight cut-off filters. Following SDS-PAGE, the enzymatic activity was validated using an in vitro transcription reaction (**Figure 5B**). A

T7 DNA template encoding a fluorescent RNA aptamer (e.g., malachite green[26]) was used, which allows monitoring of RNA production kinetics using a fluorescence plate reader, as well as comparison of transcription kinetics between different batches or designs of polymerases.

[Insert Figure 6 here]

BG-functionalized oligonucleotides were prepared using standard amine-reactive crosslinking chemistry (i.e., reacting BG-GLA-NHS esters with amine-modified oligonucleotides). Successful coupling was verified via 18% denaturing TBE-Urea PAGE (**Figure 6**). Compared to the unmodified oligonucleotide (S1), the addition of the BG moiety to the oligonucleotide increases its molecular weight and causes the BG-modified oligo (S3) to travel slower in the gel. The use of a high-percentage denaturing gel is necessary to observe this single-nucleotide difference. Denaturing PAGE analysis is also useful to characterize batch-to-batch variability in conjugation efficiency, as both conjugated and unconjugated oligonucleotide can be resolved as separate bands on the gel. If the product contains a significant amount of unconjugated oligonucleotide, a second round of chemical coupling may be applied to drive the reaction to completion.

[Insert Figure 7 here]

Following the production of the SNAP T7 RNAP and BG-modified oligonucleotide, the synthesis of the DNA-tethered RNAP was a simple one-pot mixing reaction. The resulting DNA-tethered T7 RNAP was purified from excess BG-oligonucleotides using a strong cation exchange spin column and analyzed by denaturing SDS-PAGE (**Figure 7**). As with the His-tag purification scheme described in a previous paragraph, a total of seven fractions were analyzed, including the initial flow-through (FT), three wash fractions (W1 to W3), the pooled elution fractions (E), the up-concentrated product (P), and a control containing unconjugated T7 RNAP (C). To verify successful conjugation, the gel was first stained with cyanine dye for nucleic acid, followed by Coomassie blue for the protein. As can be observed in the cyanine dye-stained gel (**Figure 7A**), the initial FT contained mostly excess BG-oligonucleotide, as well as a small portion of the DNA-tethered polymerase (i.e., RNAP-oligo) that did not bind to the cation exchange resin.

The wash fractions contained a series of fainter bands of the BG-oligonucleotides (W1–W3) at the bottom of the gel. This suggests successful removal of excess oligonucleotide. The pooled elution fractions (E) contained only the single band of RNAP-oligo caused by the binding of the cyanine dye to the oligonucleotide tether. If lane E contains bands of free oligonucleotide, more wash steps may be required to remove them from the sample. The lane containing the filtered and up-concentrated product (P) showed the same band as E, but much darker, signifying that the up-concentration procedure was successful. The protein control column contained only protein, which exhibited minimal non-specific binding to cyanine dye, seen as a faint band. The same patterns were observed in the Coomassie blue-stained gel (**Figure 7B**). A small gel mobility shift was observed when comparing the oligonucleotide-conjugated RNAP to the non-conjugated RNAP control.

[Insert Figure 8 here]

To demonstrate a method for on-demand switching of transcriptional ability in the tethered RNA polymerase system, a DNA template design that responded to a pair of nucleic-acid input strands TF_A and TF_B was used (**Figure 8A**). Transcriptional activity was monitored by measuring production of the malachite green fluorescent aptamer in both OFF (i.e., caged) and ON (i.e., uncaged) states. The amount of fluorescence signal produced at the end of the in vitro transcription is shown in **Figure 8B**, and the real-time kinetics are shown in **Figure 8C**. Here, a 336-fold activation in fluorescence signal can be observed, demonstrating robust control of polymerase activity.

FIGURE AND TABLE LEGENDS:

Figure 1: Mechanism of toehold-mediated DNA strand displacement. The toehold, δ, is a free, unbound sequence on a partial duplex. When a complementary domain (δ*) is introduced on a second strand, the free δ domain serves as a toehold for hybridization, allowing for the rest of the strand (α*) to slowly displace its competitor through a zipping/unzipping reversible reaction known as strand migration. As the length of δ increases, the ΔG for the forward reaction decreases, and displacement happens more readily.

Figure 2: Abstraction and mechanism of "tether" and "cage" polymerase complex. (**A** and **B**) An oligonucleotide tether is enzymatically labelled to a T7 polymerase through the SNAP-tag reaction. A cage consisting of a "faux" T7 promoter with a tether-complement overhang allows it to hybridize to the tether and block transcriptional activity. (**C**) When the operator (*a*b**) is present, it binds to the toehold on the oligonucleotide tether (*ab*) and displaces the *b** region of the cage, allowing transcription to occur. This figure has been modified from Chou and Shi[27]. Abbreviations: RNAP = RNA polymerase.

Figure 3: Selective programming of polymerase activity through a three-component switch activator. (**A**) When the transcription factors (TF_A and TF_B) are present, they bind to the operator domain upstream of the promoter, forming a pseudo single-stranded sequence (*a*b**) capable of displacing the cage through toehold mediated DNA displacement. (**B**) This *a*b** domain can displace the cage via TMDSD to initiate transcription. This figure has been modified from Chou and Shi[27]. Abbreviations: TF = transcription factor; RNAP = RNA polymerase; TMDSD = toehold-mediated DNA strand displacement.

Figure 4: Plasmid map for SNAP T7 RNAP. The plasmid encodes a T7 RNAP containing an N-terminal histidine tag (6x His) and SNAP-tag domain (SNAP T7 RNAP) under a lac repressor (lacI) on a pQE-80L backbone. Other features include kanamycin resistance (KanR) and chloramphenicol resistance (CmR) genes. Abbreviation: RNAP = RNA polymerase.

Figure 5: SDS-PAGE analysis of SNAP T7 RNAP expression and in vitro transcription assay. (**A**) SNAP T7 RNAP protein purification analysis, SNAP T7 RNAP molecular weight: 119.4kDa. FT = flow-through from the column, W1 = elution fractions of wash buffer containing impurities, E1–3 = elution fractions containing purified product, and DE = 10x diluted total desalted elution. 4–12% precast Bis-Tris protein gel, stain: Coomassie blue, running buffer: MES buffer, conditions:

200 V for 35 min. (**B**) An in vitro transcription assay was performed on the SNAP T7 RNAP protein by measuring production of a fluorescent aptamer over time. Transcription kinetics were monitored on a fluorescence plate reader for 2 h at 2 min intervals at 37 °C using excitation wavelength at 470 nm and emission wavelength at 512 nm. Abbreviations: RNAP = RNA polymerase; MES = 2-(*N*-morpholino) ethanesulfonic acid; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Figure 6: PAGE analysis of BG-oligonucleotide conjugation and purification. BG was conjugated on the 3'-end of the oligonucleotide through standard amine chemistry. BG-functionalized oligonucleotide conjugates were purified from excess by-products using a size exclusion chromatography spin column and analyzed on a denaturing 18% TBE-Urea PAGE following cyanine dye nucleic acid stain. An ultra-low range DNA ladder was used in this gel. S1 = oligonucleotide, S2 = pre-purification BG-oligo, S3 = post-purification BG-oligo. Abbreviations: PAGE = polyacrylamide gel electrophoresis; BG = benzylguanine; TBE = Tris-borate-EDTA; EDTA = ethylenediamine tetraacetic acid.

Figure 7: SDS-PAGE analysis of T7 RNAP-oligonucleotide conjugation and purification. A BG-modified oligonucleotide is conjugated to a T7 RNAP via SNAP-tag. The conjugates were purified from excess oligonucleotides using strong cation exchange spin columns, before being analyzed by SDS-PAGE stained with both (**A**) cyanine dye nucleic acid stain and (**B**) Coomassie blue stain. Both a protein ladder and a 10-bp DNA ladder were used in this gel. FT = flow-through from the column, W1–W3 = elution fractions of purification buffer containing impurities, E = pooled elution fractions containing purified product, P = purified product after filtration buffer exchange and up-concentration, C = SNAP T7 RNAP only as control. This figure has been modified from Chou and Shi[27]. Abbreviations: RNAP = RNA polymerase; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Figure 8: In vitro transcription assay of ON and OFF states of caged polymerase system. (**A**) Schematic depicting T7 RNAP in caged and uncaged states. (**B** and **C**) An in vitro transcription assay was performed on caged and uncaged states of the polymerase by measuring production of a fluorescent aptamer. Shown in this figure is a 336x increase in transcription rate between the ON and OFF states. Error bars indicate standard deviation (n=3). This figure has been modified from Chou and Shi[27]. Abbreviations: RNAP = RNA polymerase; TF = transcription factor.

Table 1: Lysis/equilibration buffer formula.

Table 2: Wash buffer formula.

Table 3: Elution buffer formula.

Table 4: Storage buffer formula.

Table 5: SDS-PAGE sample loading for lanes from left to right.

Table 6: In vitro transcription reaction formula with SNAP T7 RNAP (master mix).

Table 7: In vitro transcription reaction formula with wild-type (WT) T7 RNAP (master mix).

Table 8: In vitro transcription reaction formula without polymerase; buffer-only control.

Table 9: Reaction formula for benzylguanine conjugation to the oligonucleotide.

Table 10: Reaction formula for a 18% TBE-UREA denaturing PAGE.

Table 11: Denaturing PAGE sample loading for lanes from left to right.

Table 12: Reagent ratios for analytical scale coupling of BG-oligonucleotide to SNAP T7 RNAP.

Table 13: Reaction formula for the SNAP-tag labelling reaction.

Table 14: Bis-Tris PAGE (4%–12%) reaction formulas for gel lane loading samples.

Table 15: Reaction formula for elution buffer (11.1).

Table 16: Reaction formula for 5x annealing buffer.

Table 17: Reaction formula used to anneal two ssDNA templates (sense and antisense strands).

Table 18: In vitro transcription reaction formula (master mix); includes RNAP concentration.

Table 19: Reaction formula for a 12% TBE-UREA denaturing PAGE.

DISCUSSION:

This study demonstrates a DNA nanotechnology-inspired approach to control the activity of T7 RNA polymerase by covalently coupling an N-terminally SNAP-tagged recombinant T7 RNAP with a BG-functionalized oligonucleotide, which was subsequently used to program TMDSD reactions. By design, the SNAP-tag was positioned at the N-terminus of the polymerase, as the C-terminus of wild-type T7 RNAP is buried within the protein structure core and makes important contacts with the DNA template[28]. Prior attempts to modify the polymerase C-terminus have resulted in complete loss of enzymatic activity unless other compensating mutations are introduced.[29,30] In contrast, N-terminal fusions of the T7 RNAP are well-tolerated, although the choice of the fusion tag can affect polymerase activity. How different tags affect RNAP activity has not been systematically determined. The SNAP-tag was chosen because it is efficient and robust, allowing quantitative tagging for stoichiometric ratios of the protein and oligonucleotide. Alternatively, other coupling chemistries may be used to link the oligonucleotide to the polymerase, such as Ybbr[31] tags, Sortase tags[32], and SpyTags[33], or else via the introduction of unnatural amino acids bearing reactive groups. The difference in size and sequence between these tags may also affect

the activity of the resulting fusion protein, and the optimal choice for site-specific RNAP tagging merits future investigation. Finally, it is recommended to limit the length of the oligonucleotide tethered to the RNAP to < 30 nt. This is designed to reduce non-specific electrostatic interactions between the oligonucleotide with the DNA binding domain of the T7 RNAP and to facilitate purification of the oligonucleotide tethered RNAP by ion exchange chromatography.

The proposed technology described here requires the expression of a recombinant SNAP T7 RNAP, and there are two critical steps during the synthesis process that affect the overall yield of the protein product. First, the use of sonication for cell lysis can heat up the sample (section 4). To ensure efficient cell lysis and protein extraction without heat-denaturing the protein, the cell sample should be kept on ice throughout sonication and the temperature of the sonication probe monitored in between each sample. A second critical step is the buffer-exchange and up-concentration of the SNAP T7 RNAP following His-tag purification (step 4.11.2). It is important to gently pipette-wash the membrane of the centrifugal filter unit to prevent protein aggregation, which would decrease overall yield and protein functionality.

In principle, the BG-oligonucleotide reaction with SNAP-tag is quantitative at a 1:1 molar ratio. However, a range of oligonucleotide-to-polymerase stoichiometric ratios should be tested before preparing a larger batch of the material. This is because the protein concentration estimates can be inaccurate. This step may require performing SDS-PAGE of the coupling reaction at different dilutions of the BG-oligonucleotide with respect to a constant protein concentration to identify the optimal coupling ratio. During PAGE analysis, the same gel can be serially stained with two stains: a nucleic-acid stain followed by a Coomassie Blue protein stain. It is important to thoroughly wash SDS off the gel before staining with the nucleic-acid stain. This is because the SDS will trap the dye in the gel, leading to a high background signal. Furthermore, the nucleic acid stain must be performed prior to the Coomassie Blue protein stain.

While this proposed system brings together the scalability of a DNA circuit with the functionality of a protein-based transcriptional circuit, it also introduces limitations seen in transcriptional circuits. One of the many advantages of DNA computers is the stability of nucleic acids in a variety of environments. With the addition of polymerases, the tethered polymerase system must be stored under specific conditions to prevent denaturation. Furthermore, computing must occur in an environment with specific buffer conditions that allow for transcription. Although the RNA polymerase from the T7 bacteriophage is used in this demonstration, another RNA polymerase with more application-appropriate conditions may be used to circumvent this limitation.

The results show that this tethered polymerase system can be toggled between OFF (e.g., caged) and ON (i.e., uncaged) states using nucleic acid “transcription factors”. Using DNA to regulate transcription makes it possible to design transcriptional circuits at scale, including building multi-layer signal cascades and feedback. Another feature is the self-amplification of input signals received or passed through the circuit, as an activated polymerase that has hybridized to a template will continue to produce more copies of its transcript until it is stopped. This signal amplification mechanism may be exploited to amplify circuit response to low-concentration inputs. Finally, this building block can be used to implement a range of digital logics. This study

demonstrates the activation of templates via “AND logic” by designing templates that must be activated by two nucleic acid strands. Similarly, “OR logic” can be designed by making a DNA template responsive to only strand B, and introducing an “adaptor” intermediate that sequesters strand A in exchange for producing yet another copy of strand B. In this case, introducing either strand A or B as inputs into the reaction would trigger transcription of the target DNA template. The ability to rationally design a variety of circuit behaviors at scale should enable the implementation of complex molecular computing algorithms for emerging applications in disease detection, portable biomanufacturing, as well as molecular data processing and storage.

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DISCLOSURES:

There are no competing financial interests to declare by any of the authors.

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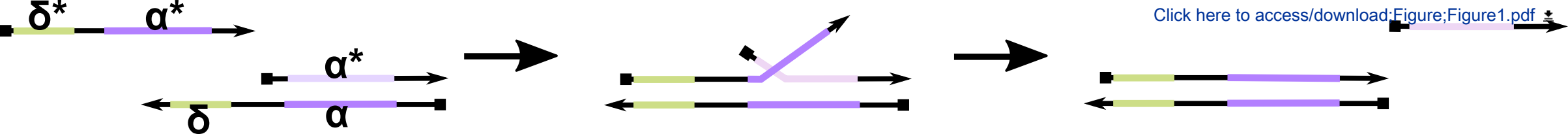

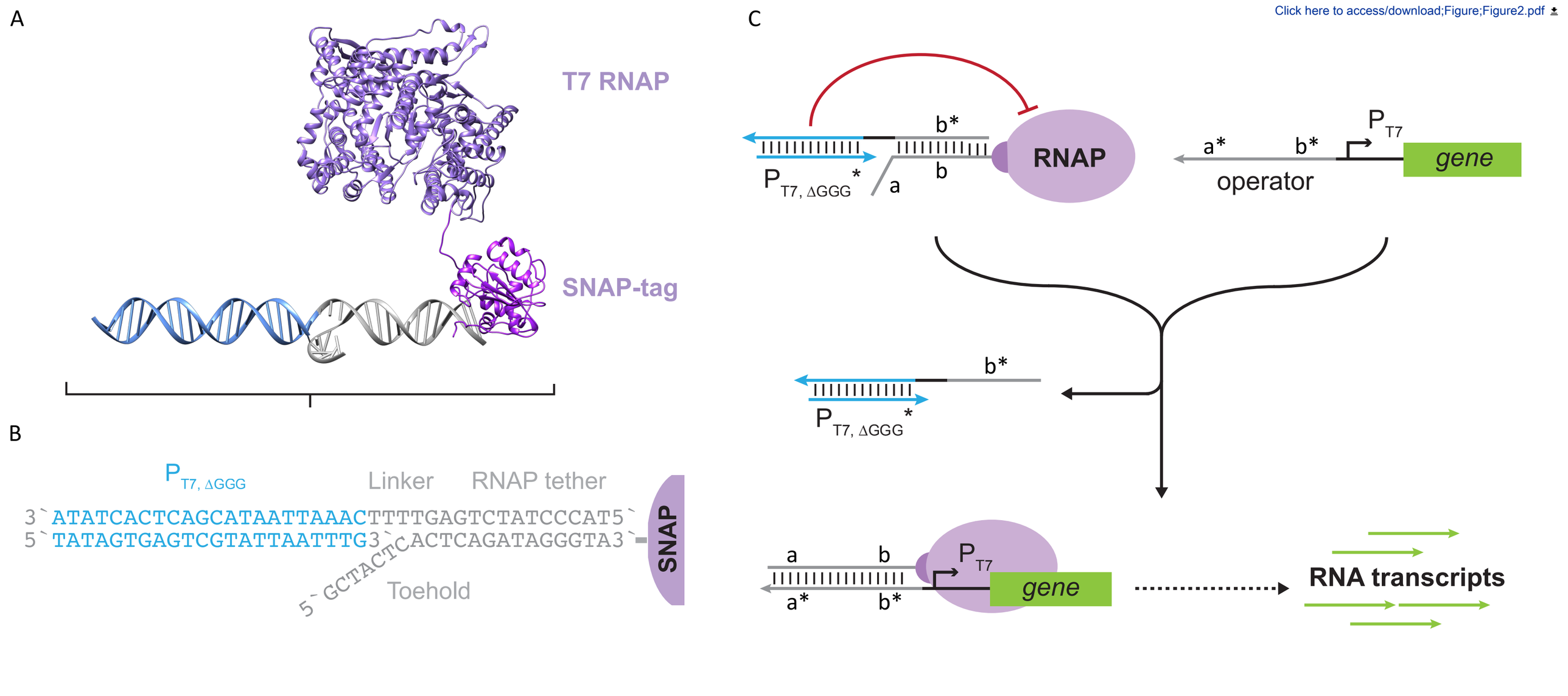

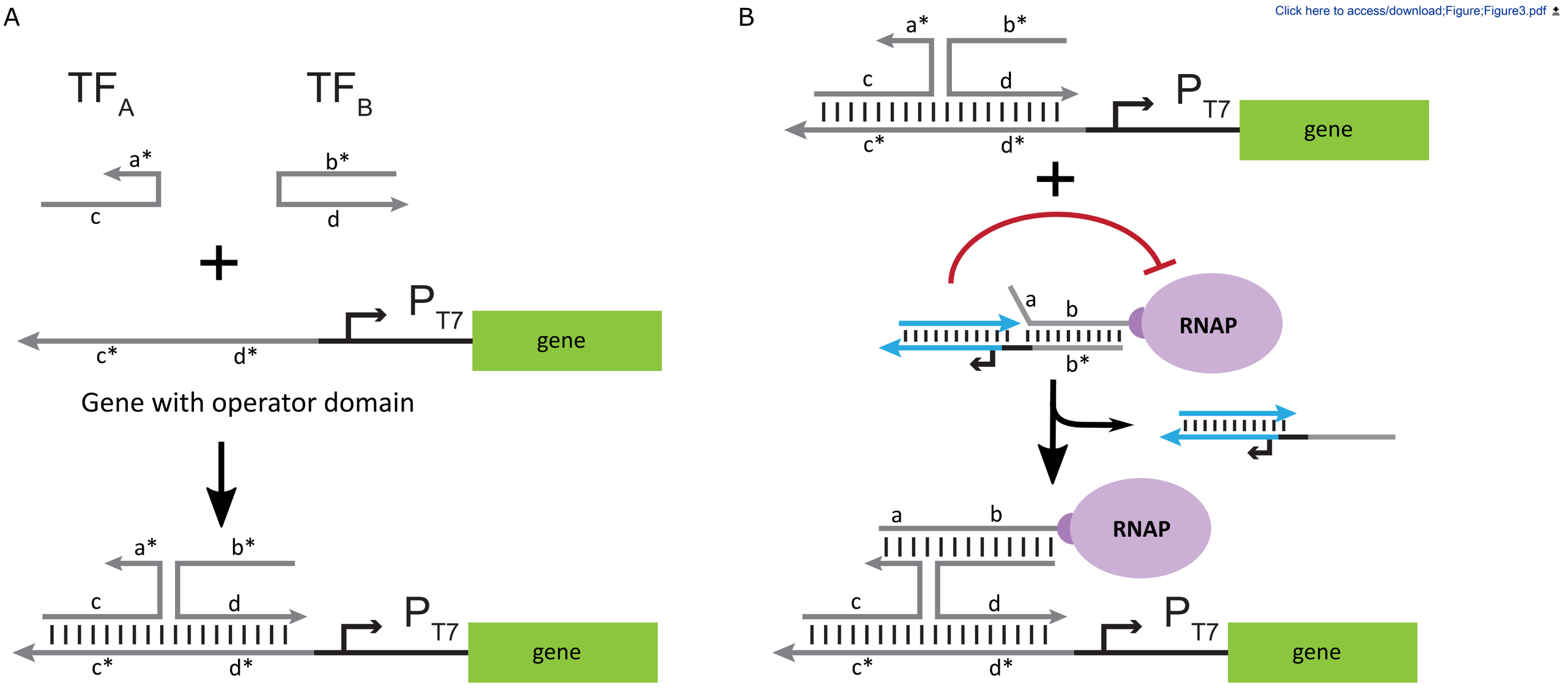

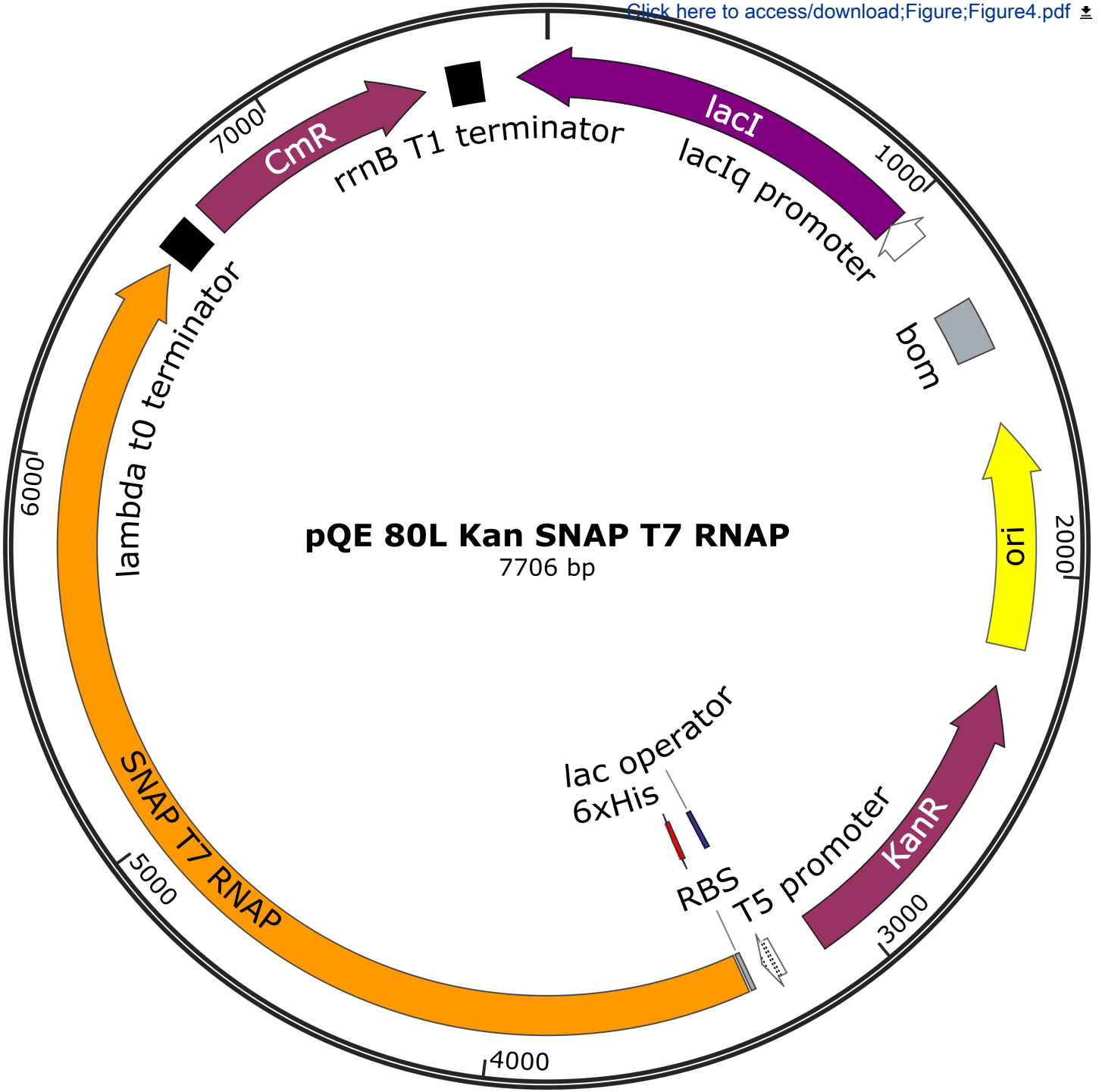

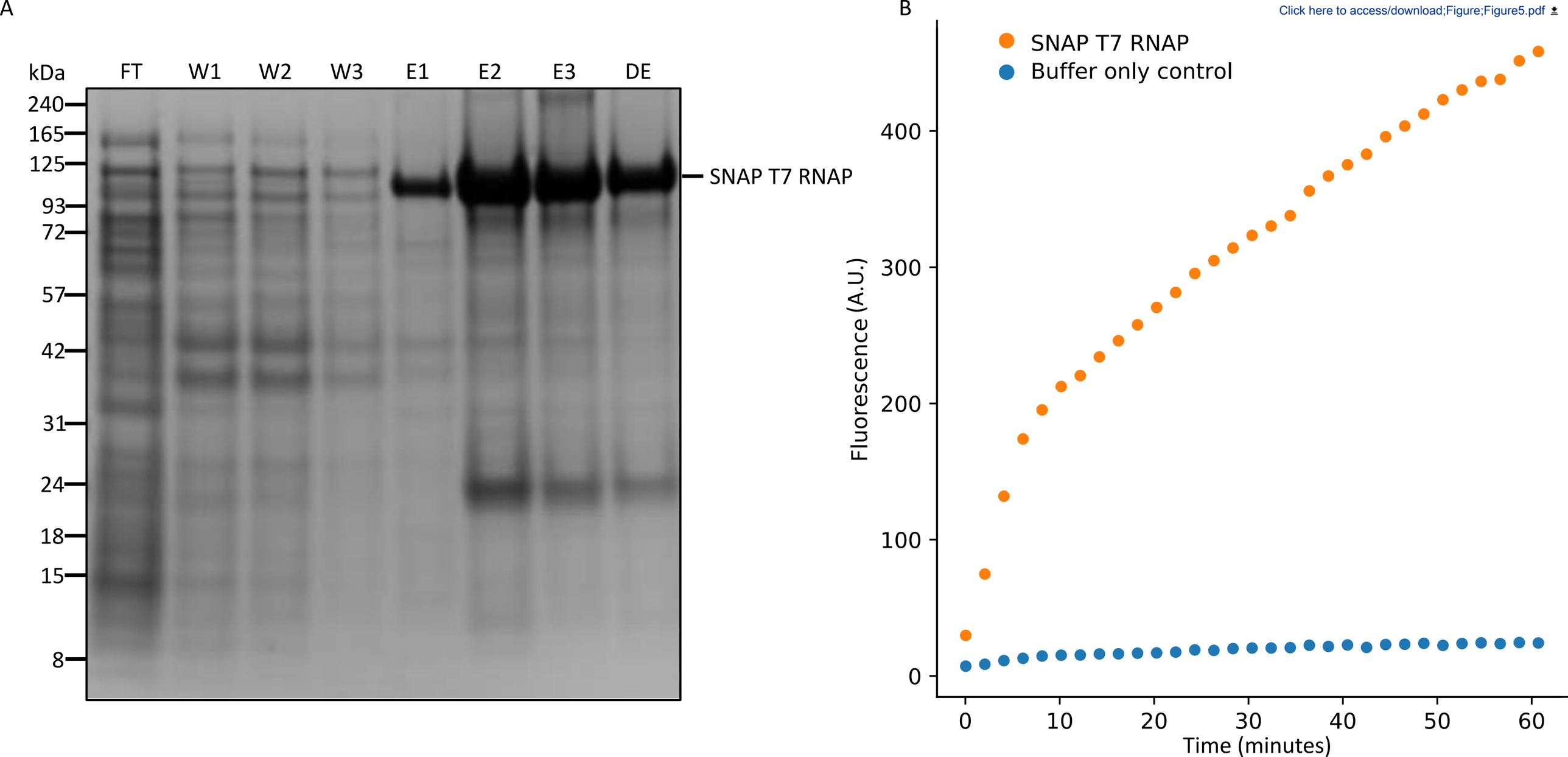
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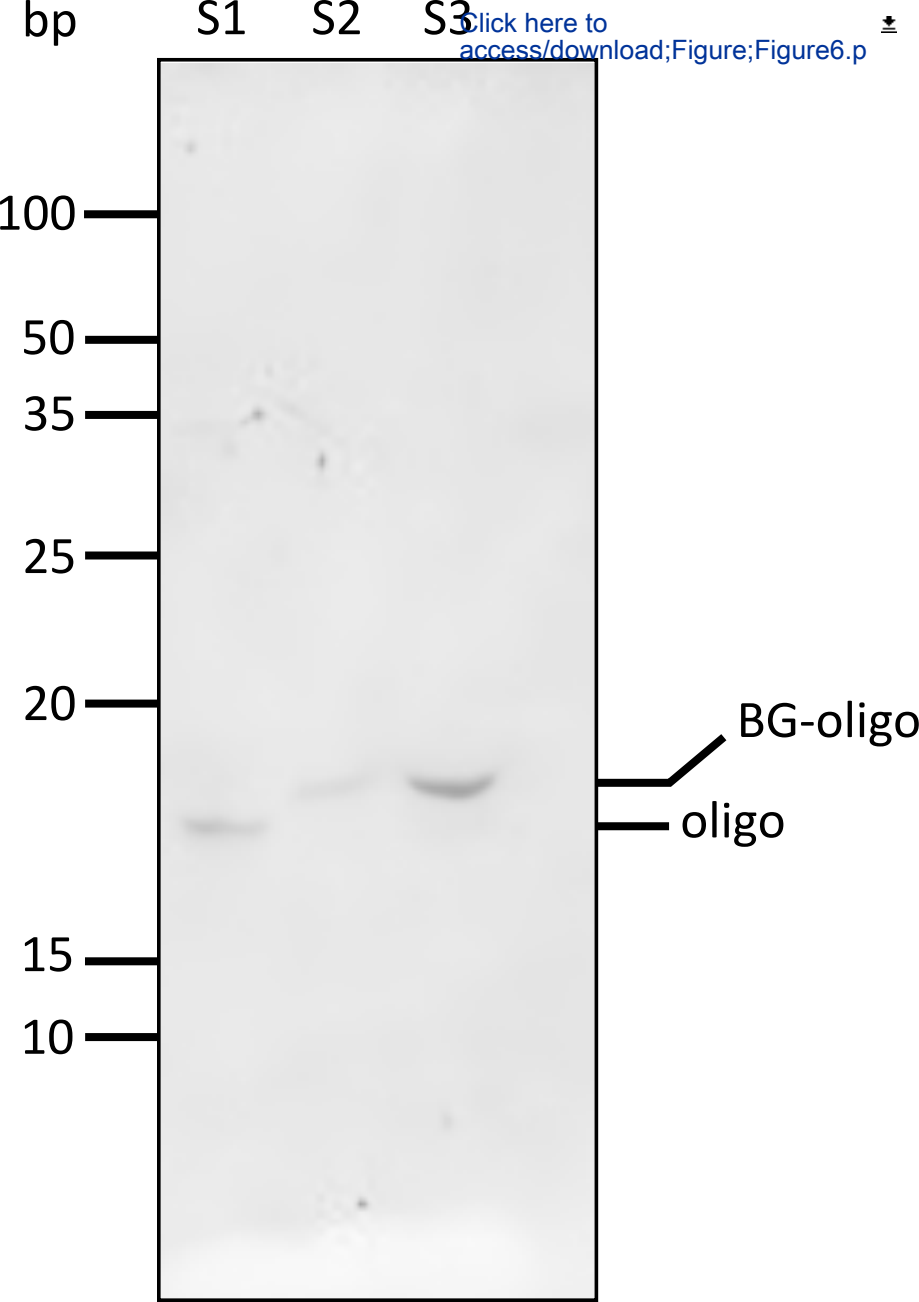

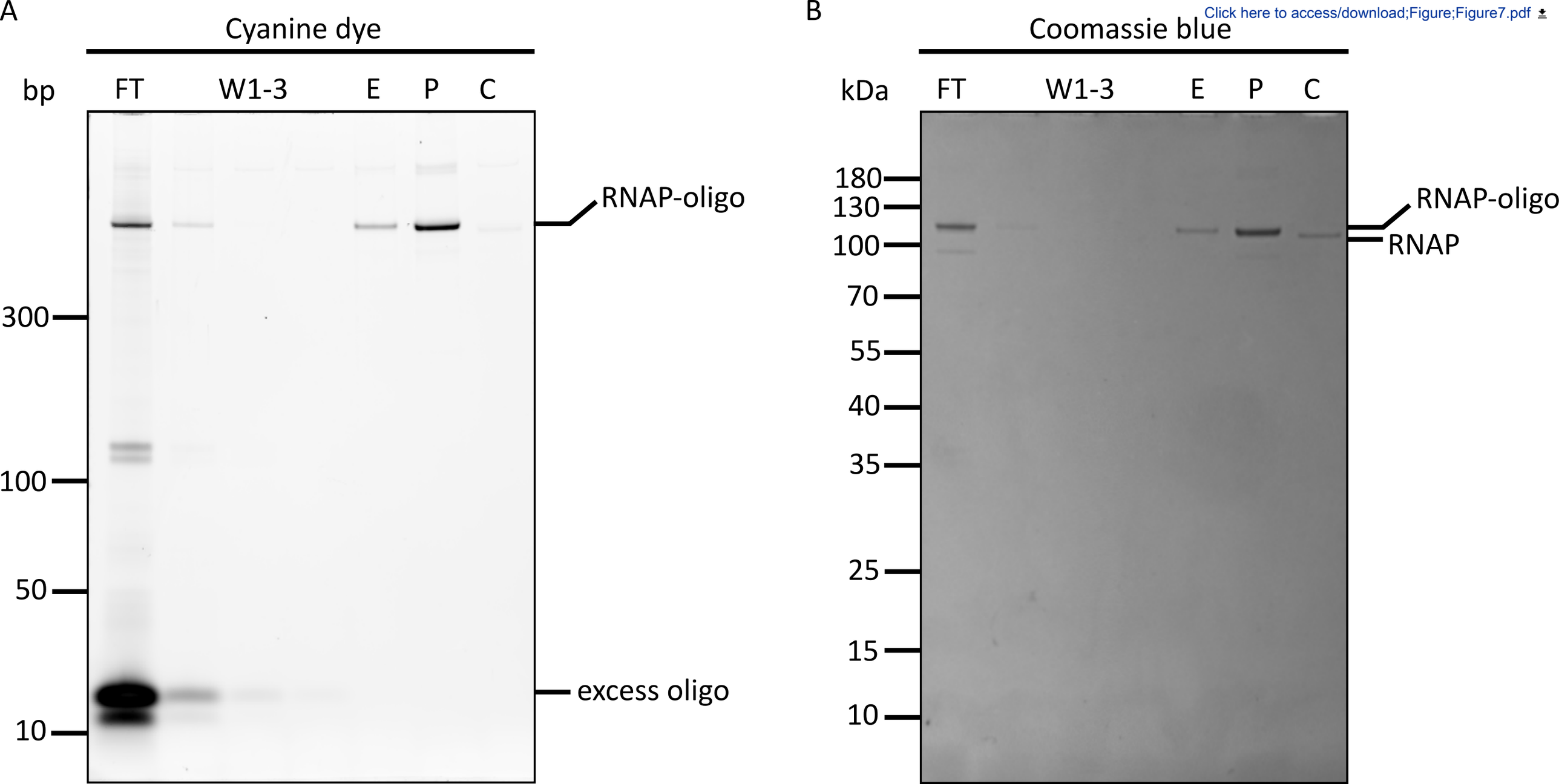

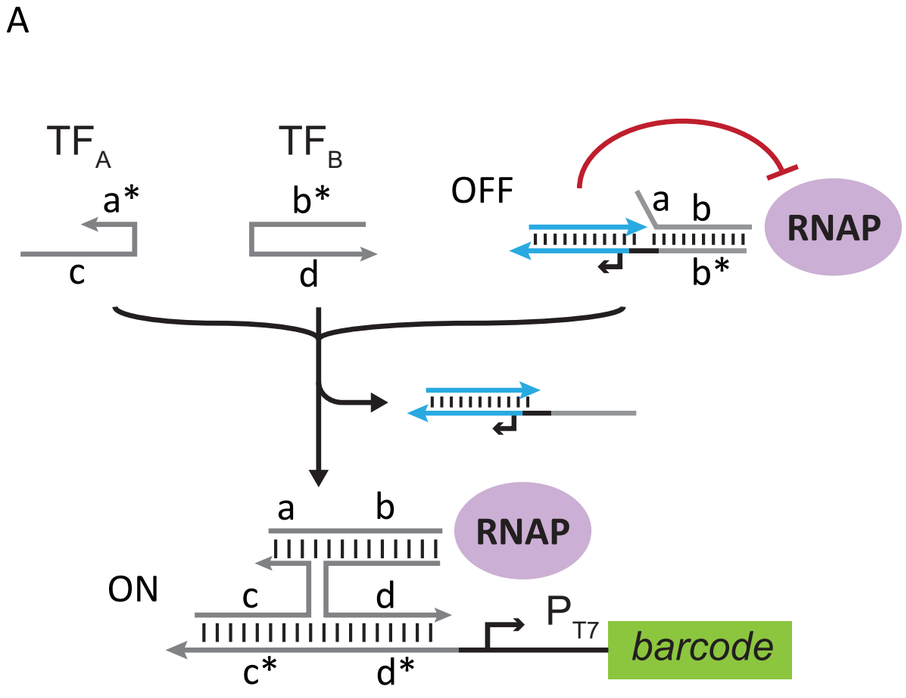

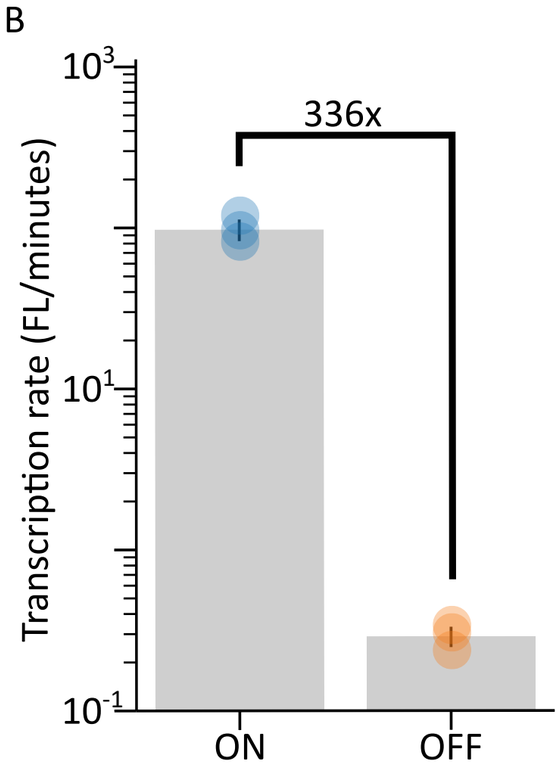

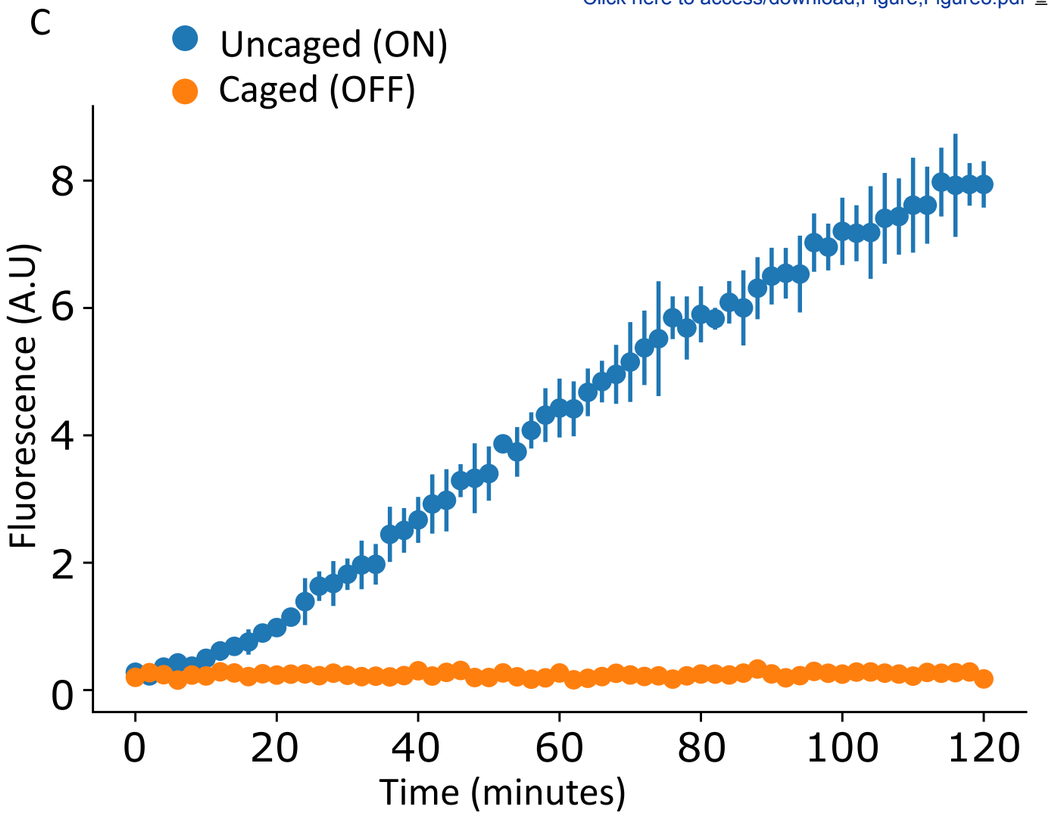

Table 1

Reagent	Initial concentration	Units	Volume	Units	Final concentration	Units
Tris	1	M	1.5	mL	50	mM
NaCl	5	M	1.8	mL	300	mM
Glycerol	100	%	1.5	mL	5	%
BME	14.2	M	10.5	µL	5	mM
ddH_2O	--	--	25.2	mL	--	--
Final volume	--	--	30	mL	--	--

Table 2

Reagent	Initial concentration	Units	Volume	Units	Final concentration	Units
Tris	1	M	1.5	mL	50	mM
NaCl	5	M	4.8	mL	800	mM
Glycerol	100	%	1.5	mL	5	%
BME	14.2	M	7.0	µL	5	mM
Imidazole	2	M	200	µL	20	mM
ddH_2O	--	--	22.2	mL	--	--
Final volume	--	--	30	mL	--	--

Table 3

Reagent	Initial concentration	Units	Volume	Units	Final concentration	Units
Tris	1	M	0.5	mL	50	mM
NaCl	5	M	1.6	mL	800	mM
Glycerol	100	%	0.5	mL	5	%
BME	14.2	M	3.5	µL	5	mM
Imidazole	2	M	1	mL	200	mM
ddH_2O	--	--	6.4	mL	--	--
Final volume	--	--	10	mL	--	--

Table 4

Reagent	Initial concentration	Units	Volume	Units	Final concentration	Units
Tris	1	M	5	mL	100	mM
NaCl	5	M	2	mL	200	mM
BME	14.2	M	140.8	µL	40	mM
Triton X-100	100	%	100	µL	0.2	%
EDTA	0.5	M	200	µL	2	mM
ddH_2O	--	--	42.56	mL	--	--
Final volume	--	--	50	mL	--	--

Table 5

Sample	Description	µL of sample	µL of loading dye
1	Protein Ladder	9	--
2	FT: Flow-through	9	3
3	W1: Wash 1	9	3
4	W2: Wash 2	9	3
5	W3: Wash 3	9	3
6	E1: Elution 1	9	3
7	E2: Elution 2	9	3
8	E3: Elution 3	9	3
9	DE: Desalted elution	9	3

Table 6

Reagent	Initial concentration	Units	Final concentration	Units	Final volume	Units
Transcription buffer	10	X	1	X	2	µL
rNTP mix	25	mM	0.5	mM	0.4	µL
DNA template	500	nM	125	nM	5	µL
SNAP T7 RNAP	500	nM	50	nM	2	µL
Nuclease-free water	--	--	--	--	10.6	µL
Total volume					20	µL

Table 7

Reagent	Initial concentration	Units	Final concentration	Units	Final volume	Units
Transcription buffer	10	X	1	X	2	µL
rNTP mix	25	mM	0.5	mM	0.4	µL
DNA template	500	nM	125	nM	5	µL
WT T7 RNAP	500	nM	50	nM	2	µL
Nuclease-free water	--	--	--	--	10.6	µL
Total volume					20	µL

Table 8

Reagent	Initial concentration	Units	Final concentration	Units	Final volume	Units
Transcription buffer	10	X	1	X	2	µL
rNTP mix	25	mM	0.5	mM	0.4	µL
DNA template	500	nM	125	nM	5	µL
Nuclease-free water	--	--	--	--	12.6	µL
Total volume					20	µL

Table 9

Reagent	Initial concentration	Units	Volume	Units	Final	Units	Final
$NaHCO_3$	1	M	25	µL	0.05	mM	1.25E-05
DMSO	100	%	284	µL	56.8	%	---
Oligo	1	mM	125	µL	0.25	µM	6.25E-08
BG-GLA-NHS in DMSO	50	mM	66	µL	6.6	mM	1.65E-06

Units	Final volume	Units
mol	500	µL
---	500	µL
mol	500	µL
mol	500	µL

Table 10

Gel volume	10 mL
Acrylamide concentration	18%
g UREA	4.8
mL of 40% acrylamide (19:1)	4.5
mL of 10x TBE	1
mL of ddH_2O	2.8
µL of TEMED	5
µL of 10% APS	100

Table 11

Sample	Description	µl sample	µl loading dye
1	L: Ultra-Low Range Ladder	3	--
2	S1: 100 nM oligo	3	3
3	S2: 100 nM oligo-BG	3	3
4	S3: 100 nM oligo-BG + Centri-Spin	3	3

Table 12

Sample	Concentration (M)	oligo:RNAP
1	2.50E-04	5:1
2	2.00E-04	4:1
3	1.50E-04	3:1
4	1.00E-04	2:1
5	5.00E-05	1:1
6	2.50E-05	1:2
7	1.68E-05	1:3
8	1.25E-05	1:4
9	1.00E-06	1:5

Table 13

Reagent	Volume	Unit
SNAP buffer	2	µL
BG-Oligo	4	µL
SNAP-T7 RNAP	4	µL
Total volume	10	µL

Table 14 Click here to access/download;Table;Table14.xlsx

Lane	Sample Description	Sample volume (µL)	SNAP buffer volume (µL)	Loading dye volume (µL)
1	Sample 1	2	4	2
2	Sample 2	2	4	2
3	Sample 3	2	4	2
4	Sample 4	2	4	2
5	Sample 5	2	4	2
6	Sample 6	2	4	2
7	Sample 7	2	4	2
8	Sample 8	2	4	2
9	Sample 9	2	4	2
10	RNAP control	2	4	2
11	Oligo control	2	4	2
12	Ladder	4		-

Table 15 Click here to access/download;Table;Table15.xlsx

Reagent	Initial concentration	Units	Volume	Units	Final concentration	Units	Final volume
Tris	1	M	50	µL	50	mM	1000
NaCl	5	M	100	µL	0.5	M	1000
ddH_2O	--	--	850	µL	--	--	1000

Units
µL
µL
µL

Table 16 Click here to access/download;Table;Table16.xlsx

Reagent	Initial concentration	Units	Final concentration	Units	volume to pipette	Units
Tris	1000	mM	25	mM	25	µL
EDTA	500	mM	5	mM	10	µL
MgCl	1000	mM	25	mM	25	µL
ddH_2O	--	--	--	--	940	µL

Table 17 Click here to access/download;Table;Table17.xlsx

Reagent	Initial concentration	Units	Final concentration	Units	Volume to pipette	Units
annealing buffer	5	X	1	X	5	µL
sense	10	µM	1	µM	2.4	µL
antisense	10	µM	1	µM	2.4	µL
ddH_2O	--	--	--	--	14.2	µL

Total Reaction Volume	Units
24	µL
24	µL
24	µL
24	µL

Table 18 Click here to access/download;Table;Table18.xlsx

Reagent	Initial concentration	Units	Final concentration	Units	Volume to pipette	Units	Total reaction volume
In vitro transcription (IVT) buffer	10	X	1	X	2.5	µL	25
rNTP mix	25	mM	1	mM	1	µL	25
Malachite Green	1	mM	40	µM	1	µL	25
RNAP	500	nM	50	nM	2.5	µL	25
DNA template	1000	nM	120	nM	3	µL	25
ddH_2O	--	--	--	--	14	µL	25

Units
µL
µL
µL
µL
µL
µL

Gel Volume	10 mL
Acrylamide concentration	12%
g UREA	4.8
ml 40% acrylamide (29:1)	3
ml 10x TBE	1
ml ddH_2O	4.3
µl TEMED	5
µl 10% APS	100

Name of Material/ Equipment	Company	Catalog Number
0.5% polysorbate 20 (TWEEN 20)	BioShop	TWN510.5
0.5M ethylenediaminetetraacetic acid (EDTA)	Bio Basic	SD8135
10 mM sodium phosphate buffer (pH 7)	Bio Basic	PD0435
10% ammonium persulfate (APS)	Sigma Aldrich	A3678-100G
100 kDa Amicon Ultra-15 Centrifugal Filter Unit	Fisher Scientific	UFC910008
100% acetone	Fisher Chemical	A18P4
100% ethanol (EtOH)	House Brand	39752-P016-EAAN
10x in vitro transcription (IVT) buffer	New England Biolabs	B9012
10x Tris-Borate-EDTA (TBE) buffer	Bio Basic	A0026
1M Isopropyl β- d-1-thiogalactopyranoside (IPTG)	Sigma Aldrich	I5502-1G
1M sodium bicarbonate buffer	Sigma Aldrich	S6014-500G
1M Tris(hydroxymethyl)aminomethane (Tris)	Sigma Aldrich	648311-1KG
1X Tris-EDTA (TE) buffer	ThermoFisher	12090015
2M imidazole	Sigma Aldrich	56750-100G
2-mercaptoethanol (BME)	Sigma Aldrich	M3148
3M sodium acetate	Bio Basic	SRB1611
40% acrylamide (19:1)	Bio Basic	A00062
4x LDS protein sample loading buffer	Fisher Scientific	NP0007
5M sodium chloride (NaCl)	Bio Basic	DB0483
5mM dithiothreitol (DTT)	Sigma Aldrich	43815-1G
6x gel loading dye	New England Biolabs	B7024S
agarose B powder	Bio Basic	AB0014
BG-GLA-NHS	New England Biolabs	S9151S
BL21 competent *E. coli*	Addgene	C2530H
BLUeye prestained protein ladder	FroggaBio	PM007-0500
bromophenol blue	Bio Basic	BDB0001
coomassie blue (SimplyBlue SafeStain)	ThermoFisher	LC6060
cyanine dye (SYBR Gold nucleic acid gel stain)	Fisher Scientific	S11494
cyanine dye (SYBR Safe nucleic acid gel stain)	Fisher Scientific	S33102
dry dimethyl sulfoxide (DMSO)	Fisher Scientific	D12345
formamide	Sigma Aldrich	F9037-100ML
glycerol	Bio Basic	GB0232

kanamycin sulfate	BioShop	KAN201.5
lysogeny broth	Sigma Aldrich	L2542-500ML
malachite green oxalate	Sigma Aldrich	2437-29-8
N,N,N'N'-Tetramethylethane-1,2-diamine (TEMED)	Sigma Aldrich	T9281-25ML
NuPAGE MES SDS running buffer (20x)	Fisher Scientific	LSNP0002
NuPAGE Novex 4-12% Bis-Tris gel 1.0 mm 12-well	Life Technologies	NP0322BOX
oligonucleotide (cage antisense)	IDT	N/A
oligonucleotide (cage sense)	IDT	N/A
oligonucleotide (malachite green aptamer antisense)	IDT	N/A
oligonucleotide (malachite green aptamer sense)	IDT	N/A
oligonucleotide (Transcription Factor A)	IDT	N/A
oligonucleotide (Transcription Factor B)	IDT	N/A
oligonucleotide with 3’ Amine modification (tether)	IDT	N/A
Pierce strong ion exchange spin columns	Fisher Scientific	90008
plasmid encoding SNAP T7 RNAP and kanamycin resistance genes	Genscript	N/A
protein purification column (HisPur Ni-NTA spin column)	Fisher Scientific	88226
rNTP mix	New England Biolabs	N0466S
Roche mini quick DNA spin column	Sigma Aldrich	11814419001
Triton X-100	Sigma Aldrich	T8787-100ML
Ultra Low Range DNA ladder	Fisher Scientific	10597012
urea	BioShop	URE001.1

	Comments/Description
Tablets used to make 10 mM buffer	

TATAGTGAGTCGTATTAATTTG
TCAGTCACCTATCTGTTTCAAATTAATACGACTCACTATA
GGATCCATTCGTTACCTGGCTCTCGCCAGTCGGGATCCTATAGTGAGTCGTATTACAGTTCCATTATCGCCGTAGTTGGTGTACT
TAATACGACTCACTATAGGATCCCGACTGGCGAGAGCCAGGTAACGAATGGATCC
AGTACACCAACTACGAGTGAG
TCAGTCACCTATCTGGCGATAATGGAACTG
GCTACTCACTCAGATAGGTGACTGA/3AmMO/

custom gene insert

Dear Editor,

We thank you and the reviewers for the helpful feedback. Please see below for a point-by-point response to editorial and reviewer comments:

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We have proofread the manuscript and defined all abbreviations at first use.

2. Please revise the following lines to avoid overlap with previously published work: 3.4-3.5; from note after 3.5 (from "for the 0.2,..") to 3.7 ("...extract to the ")

The following concern has been addressed. Changes have been made to the protocol for steps 3.5 through to 3.8.

3. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have added these details to our protocol steps.

4. Note after 1.2: please cite a reference for the plasmid and related details. Please also provide one or more figures that will help readers understand what the plasmid and the proteins (His-tagged etc) look like.

We have cited a reference for the plasmid and included a plasmid map in the revised Figure 4. We have also included a schematic of the SNAP T7 RNAP iin Figure 2A.

5. What is the composition of the storage buffer, equilibration buffer, wash buffer etc?

We have added the composition of the buffers in the buffer preparation protocol section. They are also listed in the following tables:

Table 1. Lysis/equilibration buffer formula.

Table 2. Wash buffer formula.

Table 3. Elution buffer formula.

Table 4. Storage buffer formula.

Table 15. Reaction formula for elution buffer (11.1).

Table 16. Reaction formula for 5X annealing buffer.

6. To clarify, is the highlighted portion of your protocol a specific example for the video that is derived from the unhighlighted part of your protocol?

The highlighted portion of the protocol is the portion we wish to demonstrate in the video.

7. Please include table legends in the figure and table legends section.

Table legends have now been included in the figure and table legends section.

8. As we are a methods journal, please add limitations of your technique to the Discussion.

We have added a discussion of the limitations to the Discussion, which we include here for reference:

"While our proposed system brings together the scalability of a DNA circuit with the functionality of a protein-based transcriptional circuit, it also introduces limitations seen in transcriptional circuits. One of the many advantages of DNA computers is the stability of nucleic acids in a variety of environments. With the addition of polymerases, the tethered polymerase system must be stored in specific conditions to prevent denaturation. Furthermore, computing must occur in an environment with specific buffer conditions that allow for transcription. While the RNA polymerase from the T7 bacteriophage is used in this demonstration, another RNA polymerase with more application-appropriate conditions may be used to circumvent this limitation."

9. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references and do not abbreviate journal names.

The references have been corrected to the appropriate format.

10. Please sort the Materials Table alphabetically by the name of the material.

The Materials Table has been readjusted to sort materials alphabetically.

Reviewers' comments:

Reviewer #1:

In this work, Lee et al. develop a building block for molecular computing. It consists of an engineered T7 polymerase covalently fused to a user-designed oligonucleotide tether. This design allows regulating the enzymatic activity of the polymerase via toehold-mediated DNA strand displacement (TMDSD) reactions. The paper is well suited for JoVE as it presents an interesting methodology to enable programmable DNA computing. However, the text must be improved to increase clarity:

Major concerns:
- Paragraphs 5-7 of the Introduction aim to explain the key ideas and concepts of the proposed strategy, but the explanation is convoluted and not clear enough. Improving these paragraphs will positively affect the understanding of the strategy.

We have edited paragraphs 5-7 of the manuscript for a clearer explanation of the proposed strategy. We include the revised paragraphs here for review:

"Here we introduce a novel building block for molecular computing that combines the functionalities of transcriptional circuits with the scalability of DNA-based circuits. This building block is a T7 RNAP covalently attached with a single-stranded DNA tether (Figure 2A). To synthesize this DNA-tethered T7 RNAP, we first produced the polymerase fused to an N-terminal SNAP-tag[24] via recombinant expression in *E. coli*. The SNAP-tag is then reacted with an oligonucleotide functionalized with the BG SNAP-Tag substrate. The oligonucleotide tether allows us to position molecular guests in close proximity to the polymerase via DNA hybridization. One such guest is a competitive transcriptional blocker that we refer to as a "cage", which consists of a "faux" T7 promoter DNA duplex with no gene downstream (Figure 2B). When bound to the RNAP via its oligonucleotide tether, the cage stalls polymerase activity by outcompeting other DNA templates for RNAP binding, rendering the RNAP in an "OFF" state (Figure 2C). To activate the polymerase to an "ON" state, we designed T7 DNA templates with single-stranded "operator" domains upstream of the T7 promoter of the gene. The operator domain (i.e., domain *a*b** Figure 2C) can be designed to displace the cage from the RNAP via TMDSD and position the RNAP proximal to the T7 promoter of the gene, thus initiating transcription. Alternatively, we also designed DNA templates where the operator sequence is complementary to auxiliary nucleic-acid strands that we refer to as "artificial transcription factors" (i.e., TF_A and TF_B strands in Figure 3A). When both strands are introduced into the reaction, they will assemble at the operator site, creating a new pseudo-contiguous domain *a*b**. This domain can then displace the cage via TMDSD to initiate transcription (Figure 3B)."

- A concept that is key to the methodology developed in the manuscript is TMDSD. Whereas the authors state in the Introduction that it is a well-established motif in the field of dynamic DNA nanotechnology, JoVE aims to publish self-contained articles that clearly present methods. Therefore, an introduction briefly presenting TMDSD will greatly improve the clarity of the manuscript.

We thank the reviewer for this suggestion, and we have added an introduction to TMDSD and revised Figure 1 to help illustrate the mechanism.

Minor concerns:
- Figures 1A, 6A, and 6C are never mentioned in the main text.

We have now added references to these figures in the main text

Text on pages 12-14 is highlighted in yellow, what does it mean?

JoVE requires the authors to highlight specific texts in the manuscript that they wish to include for video production.

Reviewer #2:
The paper by Lee et al. demonstrates how programmable self-assembly of nucleic acid concepts and tools can shape the enzymatic activity of the phage-derived T7 RNA polymerase (RNAP) means to tune the dynamics of synthetic gene regulatory networks. They engineered a synthetic oligonucleotide-tethered T7 RNAP enzyme that, based on nucleic-acid strand displacement, can be used in programmable transcription in vitro applications. Also, they generated auxiliary nucleic acid assemblies that can be used as "artificial transcription factors" to regulate interactions between RNAp-T7-DNA templates.

The work is well-planned and presented, but it is poorly written; many sentences in the Introduction and Discussions of the manuscript require English revisions. In its present form, provided the authors revise the significant points and concerns highlighted here, I recommend this work to be published in the Journal of Visualized Experiments.

The authors should revise the manuscript following the comments below and consider citing relevant publications to bring light to the relevance of the work. They should also consider clarifying the novel mechanisms' schematics to represent ON/OFF and Cage/Uncaged states of the system within the drawings. The definition in Figure1, for example, should bring the concept of ON/OFF tool and how one can make use of this mechanism straight away.

I added a few examples of revisions to be considered, starting from the introduction section, and added some points to help the author address the minor problems of the manuscript:

1-The first two sentences, for example, should give the readers the past critical publications in the field:
"DNA computing uses a set of designed oligonucleotides as the medium for computation. These oligonucleotides are programmed with sequences to dynamically assemble according to the user-specified logic and in response to specific nucleic-acid inputs."

We thank the reviewer for these helpful comments. We have added citations to support this statement.

2-There should be a sentence indicating the scientific relevance of the work and why novel regulatory mechanisms need to be developed for in vitro transcription regulation?

We have added justification for development of nucleic-acid-based transcription regulation.

3-Please add two examples (with citations) for systems that can interface with bio-systems in the following paragraph:

"Compared to silicon-based computers, the advantages of DNA computers include their ability to interface directly with biomolecules, operate in solution in the absence of a power supply, as well as their overall compactness and stability."

We have cited Lopez, Wang & Seelig and Pardee et al as DNA computers which interface directly with biological molecules.

4-These sentences are a bit confusing, as they require revisions for English:

"Compared to self-assembly-based DNA circuits, the de novo synthesis of RNA polymers involved in vitro transcriptional circuits also enables an efficient means of in situ signal amplification and signal cascading. However, one pitfall of current transcriptional circuits is the lack of scalability, and this is because their regulation requires the use of orthogonal protein-based transcription factors, which are difficult to design."

" The proposed computing architecture is more scalable than previous designs17 because it has no sequence constraints. Coupled with a programmable TMDSD activation switch, we propose this building block will enable the construction of large cascading molecular circuits for demanding applications such as multi-gene expression analysis and molecular information processing."

We have re-written these paragraphs for clarity. They are appended below for reference:

“Synthetic gene circuits, such as transcriptional gene circuits, are also capable of computation[21–23]. These circuits are regulated by protein transcription factors, which activate or repress transcription of a gene by binding to specific regulatory DNA elements. Compared to DNA-based circuits, transcriptional circuits have several advantages. First, enzymatic transcription has much higher turnover rate than existing catalytic DNA circuits, thus generating more copies of output per single copy of input, providing more efficient means of signal amplification. In addition, transcriptional circuits can produce different functional molecules, such as aptamers or messenger RNA (mRNA) encoding for therapeutic proteins, as computation outputs, which can be exploited for different applications. However, a major limitation of current transcriptional circuits is their lack of scalability. This is because there currently exists a very limited set of orthogonal protein-based transcription factors and de novo design of new protein transcriptions factors remains technically challenging and time-consuming.”

5-What is the actual advantage of a caged/non-caged T7-RNAp instead of selecting the concentration and timing of a T7 promoter by merely adding a conventional T7-RNAp in the system at appropriate moments? The authors should discuss how the system proposed here differs from a regular cell-free TXTL system, for example, containing T7-RNAP with a controllable injection of this particular enzyme (by a syringe pump, externally) into a tube containing DNA template activated by T7-promoter to control the expression of any gene cassette.

Aside from transcription at a controlled timepoint, the caged/non-caged system has other unique advantages: (i) it enables selective transcription of a single template from a pool of templates; (ii) it enables autonomous signal cascading, where the output of one template activates the another; (iii) similar to the previous point on cascading. this makes it possible to implement molecular circuits with feedback. We have mentioned these features in the last paragraph of the introduction.

6-Please add a sentence to introduce the SNAP-tag mechanism in the paragraph:

"To synthesize the DNA-tethered T7 RNAP, we first produced the polymerase fused to an N-terminal SNAP-tag via recombinant expression in E. coli."

We have added this sentence in our introduction.

In the representative results section, please address the following points:

1-In Fig-1C and Fig2, although there is a red arrow representing the switching aspect, the schematics of the states ON and OFF of the mechanism are not exact, lacking the demonstration of how this mechanism switches transcription active to inactive.

The figures have now been revised as Figure 2C and Figure 3A. We have discussed these differences individually in the introduction paragraph as appended above.

2-In the plot presented in Fig3-B, it is unclear how a line is obtained from multiple experimental data-points in time. Is this a line-fit of any sort? Or simply the connection of the data-points? Please make the actual data-point relevant in the plot (as small solid circles or diamonds). Most importantly, there should be error bars representing biological replicates of the results. The same revision should be considered for Fig6-C

These two figures have now been revised as Figures 5-B and 8-C. The line is simply a connection between data-points. We have included data-points in the plot. The kinetic traces are representative plots, and the independent replicates have been included in the endpoint.

3-Similar to Fig5-B, Fig3 should also explain the fold-change difference expected for the novel transcription mechanism regulation.

These two figures have now been revised as Figures 5-B and 8-C. Figure 5-B is a representative plot of transcription kinetics from a single batch of recombinantly expressed SNAP T7 RNAP. We use this to verify that the expressed proteins are functional.

4-The system presented in Fig2 or Fig6 with two transcription factors does not inform very clear how one or two TFs can act together to either activate or inactivate genes downstream of the process. Namely, it is not clear whether the mechanism of activation is the fact that now T7-RNAp is release to drive transcription, while TFs are removed from the system or the fact that TFs are attached to T7-RNAP, there is an increase in the binding specificity and affinity to T7-promoters, acting as natural sigma factors, which would not be possible otherwise. I see some attempt to explain the mechanism in the caption of Figures 1 and 2, but their schematics do not show the mechanism precisely. I suggest better adaption of the re-printed figures, perhaps the creation of your schematics this time.

*"Fig2. … when the transcription factors (TFA and TFB) are present, they bind to the anchor region upstream of the promoter, forming a pseudo-single-stranded sequence (a*b*), capable of displacing the cage through toehold mediated DNA displacement."*

We have re-adapted Figure 2 to show binding domains and mechanism more clearly. This is now Figure 3 in the revised manuscript. We have kept Figure 6 the same for brevity and clutter.

4-Fig5-C is hard to interpret the test and the result intended. Please add a legend in the gel lanes to inform what they refer to. If it is the case, please add some comparison indicating the results that should be presented (e.g., comparing the desired product and at least one control) to prove your point. However, the gel itself does not look great. I strongly suggest replacing this gel (perhaps repeat this result) to add a better-looking figure of the gel.

The intention of this gel figure was to illustrate how the SDS in the running buffer of the gel interacts poorly with cyanine nucleic acid staining dye and the importance of the washing step of the protocol. In light of the reviewer's suggestion, we have removed this figure in favour of stressing the importance of the washing step instead within the protocol itself.

Reviewer #3:

I understand that the paper need not be scientifically novel, but I assume it should still be useful. Here the manuscript and all figures are almost perfect copies of the original research paper, and the protocols are overly simple molecular biology routines: recombinant protein expression with histag purification, gel purif, and a protocol to couple benzylguanine on oligos (diregarding the fact that this moiety is widely available as a modiciation on commercial oligos).

We thank the reviewer for this feedback. Our goal was to outline a methodology for synthesizing and characterizing a DNA-conjugated enzyme, and to highlight the potential of using this strategy to control enzymatic activity and design *in vitro* gene circuits.

Reviewer #4:

Manuscript Summary:

The manuscript from Lee and co-authors focuses on DNA-based nanotechnology to program the enzymatic activity of the phage derived T7 RNA polymerase (RNAP) and to build scalable synthetic gene regulatory networks.

The approach is elegant and the protocol is well written and suitable for publication.

I would just suggest to include some relevant citations and comment more in depth about the perspective application of this tool.

Minor Concerns:

1. In the following sentence" Over the past thirty years, increasingly complex DNA computational circuitries have been demonstrated, such as various digital logic cascades, chemical reaction networks, and neural networks1-3. " It's worth to mention also the importance of tight predictability of synthetic networks (https://doi.org/10.1007/978-1-61779-412-4_4; https://doi.org/10.1007/s11047-018-9715-9), and of tools for robust functionality (e.g. computational sequence design for various applications https://doi.org/10.3389/fbioe.2014.00041; https://doi.org/10.1007/978-1-4939-7223-4_18; https://doi.org/10.1016/j.asoc.2013.06.010).

We have added mentions of mathematical predictions and computation tools (and their citations).

2. "The ability to rationally design a variety of circuit behaviours at scale should enable the implementation of complex molecular computing algorithms for applications such as disease detection, portable biomanufacturing, as well as molecular data processing and storage. "
It would be interesting if the authors could add few more insights on the use of this approach with some examples of relevant, recent works in that direction.

We have included citations for recent works in disease detection, biomanufacturing and data storage.