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DNA aptamers targeting *P. aeruginosa* RNAP

Hari Timilsina[1†], Dmytro Kompaniiets[2†], Satya Prakash Arya[1], Rick Mason Postema[1], Raunak Jahan[1], Andrew Michael Reynolds[1], Siddhartha Kalpa Samadhi Thennakoon[1], Bin Liu[2*] and Xiaohong Tan[1*]

[1]Department of Chemistry and Center for Photochemical Sciences, Bowling Green State University

[2]Section of Transcription & Gene Regulation, The Hormel Institute, University of Minnesota, Austin, MN, USA

†These authors contributed equally to this work.

*To whom correspondence should be addressed: tanx@bgsu.edu; liu00794@umn.edu

Supporting Information

Materials and Methods

Chemicals and reagents. All proteins used in this study were provided by a collaborator. All aptamers and other nucleic acids were sourced from Integrated DNA Technologies, Inc. (www.idtdna.com) as lyophilized powders and were reconstituted in nano-pure water upon arrival. Ni-NTA magnetic beads (Ref# 062N-A) were acquired from G-Biosciences and used at the necessary dilutions for the experiments. All chemicals were obtained from Sigma-Aldrich unless stated otherwise.

The RNAP core and σ^{70} expression. Plasmids encoding *P. aeruginosa* core RNAP and *P. aeruginosa* σ^{70} factor RpoD were provided by Dr. Yangbo Hu at the Chinese Academy of Sciences. Briefly, the RNAP subunit genes were cloned into vector pET-28a by individually inserting each of the five subunits with T7 promoter and RBS sequence in front of each subunit. The 6x His-tag was put in the C-terminus of β' subunit. The *P. aeruginosa* RpoD was also cloned and expressed using pET-28a as an MBP fusion protein.

Both core RNAP and RpoD were overexpressed in *E. coli* BL21 (DE3) (ThermoFisher Scientific) at 20°C overnight. Induction was performed at OD600 in a range between 0.6-0.8 using 0.3 mM final concentration of Isopropyl β-d-1-thiogalactopyranoside (IPTG). Cells were collected, frozen, and stored at -80°C until needed.

RpoD purification. RpoD MBP fusion contains a 6xHis-tag. For His-tag affinity purification cell pellet was resuspended in lysis buffer (20mM Tris HCl pH 8.0, 200mM NaCl, 5mM Imidazole + Protease Inhibitor Cocktail (complete ™)) and sonicated for 40 cycles: 30-second pulse, 1 minute rest on ice at 60% power output. Lysate was centrifuged twice at 20Kx RPM for 20 and 15 minutes respectively and supernatant was further filtered through a 0.22 μM filter (VWR International). Protein-containing solution was applied onto the 5ml HP HisTrap column (Cytiva) preequilibrated with the Buffer A (20mM Tris HCl pH 8.0, 200mM NaCl, 5mM Imidazole). Protein was eluted using step elution. After the column wash with 30ml of Buffer containing 25mM Imidazole, protein was eluted with 300mM Imidazole over 30 ml. Peak fractions were analyzed with SDS-PAGE. Protein-containing fractions were combined and concentrated, then diluted with 50mM Tris-HCl pH 8.0 to dilute imidazole and incubated with TEV protease to remove MBP and 6His-tag at 4°C overnight (**Figure S1A**). The protein solution was diluted twice more with 20mM Tris-HCl pH 8.0 200mM NaCl and run through HisTrap column again to remove uncut protein. Flowthrough fraction containing RpoD was diluted to the final NaCl concentration of 50mM and applied to 5ml Heparin column (Cytiva) preequilibrated in buffer B (50mM Tris HCl pH 8.0, 50mM NaCl). Protein was eluted over the 100ml NaCl gradient (50-1000mM). Protein-containing fractions were combined and concentrated using a centrifugal filter unit (MilliporeSigma™ Amicon™ Ultra-15 MWCO 30 kDa). Concentrated protein was applied onto Superdex® 200 Increase 10/300 GL size exclusion column (Cytiva) equilibrated with Buffer C (40mM Tris HCl pH 8.0, 300mM NaCl). Protein-contained fractions were concentrated, aliquoted, flash-frozen, and stored at -80°C until needed.

Core RNAP purification. The β' subunit within core RNAP contains a 6xHis-tag. For His-tag affinity purification cell pellet was resuspended in lysis buffer (50mM Tris HCl pH 8.0, 300mM NaCl, 5mM Imidazole + Protease Inhibitor Cocktail (complete ™)) and sonicated for 40 cycles: 30-second pulse, 1 minute rest on ice at 60% power output. Lysate was centrifuged twice at 20Kx RPM for 20 and 15 minutes respectively and supernatant was further filtered through a 0.22 μM filter (VWR International). The protein-containing solution was applied onto the 5ml HP HisTrap column (Cytiva) preequilibrated with

Buffer A (50mM Tris HCl pH 8.0, 300mM NaCl, 5mM Imidazole). Protein was eluted using step elution. Firstly, the column was washed with 30ml of Buffer containing 25mM Imidazole, then the protein was eluted with 300mM Imidazole over 30 ml. Peak fractions were analyzed with SDS-PAGE. Protein-containing fractions were combined and diluted with 50mM Tris-HCl pH 8.0 to the final NaCl concentration of 50mM. Diluted fractions were applied to 5ml Heparin column (Cytiva) preequilibrated in buffer B (50mM Tris HCl pH 8.0, 50mM NaCl). Protein was eluted over the 100ml NaCl gradient (50-1000mM). Protein-containing fractions were combined and concentrated using a centrifugal filter unit (MilliporeSigma™ Amicon™ Ultra-15 MWCO 30 kDa). Concentrated protein was applied onto Superdex® 200 Increase 10/300 GL size exclusion column (Cytiva) equilibrated with Buffer C (40mM Tris HCl pH 8.0, 300mM NaCl). Protein-contained fractions were concentrated, aliquoted, flash-frozen, and stored at -80°C until needed (**Figure S1B)**.

PA RNAP holoenzyme assembly. Core RNAP and RpoD were mixed at 1:3 molar ratio respectively in buffer 20 mM Tris 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT and incubated at 37°C for 10 minutes. Reaction mix was applied on Superose® 6 Increase 10/300 GL size exclusion column (Cytiva) equilibrated in the same buffer. Fractions containing the holoenzyme were further used.

SELEX procedure. We conducted the DNA-SELEX experiment using the RNAP protein as the target. The oligonucleotide library was obtained from IDT and consists of 40 random nucleotides flanked by constant primer sequences (Figure S5). For the first round of the selection, 100 pmol of the spike-protein and 1 µL of nickel nitrilotriacetic acid (Ni-NTA) beads (Ref# 062N-A; G-bioscience) were diluted into 100 µL of SELEX buffer (PBS-Mg buffer, PBS with 1mM $MgCl_2$, pH 7.4) and incubated at room temperature (RT), rotating for 1 h. Meanwhile, 3 nmol of DNA library was diluted into 100 µL of PBS-Mg and treated at 95 °C for 5 min, on ice (or 4 °C) for 5 min, RT for 5 min, and placed in ice. When 1 h of incubation was completed, the protein-bead (P-B) complex was washed two times with 200 µL SELEX buffer and combined with a heat-treated DNA library, with 1 µL of 100 times concentrated tRNA and incubated for 1 h at RT with rotation. After incubation, the protein-bead-library (PBL) complex was washed two times with 200 µL SELEX buffer to remove the unspecific library. After washing, the bound library was eluted 2 times by 30 µL of hot water at 95°C. The selected library was amplified by polymerase chain reaction (PCR). For the first round of selection, the PCR mixture contained 60 µL of the library, 39 µL of nuclease-free water, 100 µL of 2 × PCR solution, and 1 µL of Easy Taq polymerase. The 50 µL of the PCR mixture was loaded into each PCR tube and amplified in the conditions of 2 min at 95°C; 9 cycles of 45 s at 95°C; 30 s at 54°C; 30 s at 72°C, 2 min at 72°C. After completing the PCR, all the PCR products were collected in a tube. To optimize the PCR cycle number for bulk amplification, 5 µL of the PCR product, 119 µL water, 125 µL of 2 × PCR solution, and 1.25 µL Easy Taq polymerase were mixed in a tube and then distributed equally (50 µL) into 5 PCR tubes. Amplification conditions were 2 min at 95°C; 3-11 cycles of 45 s at 95°C; 30 s at 54°C; 30 s at 72°C, 2 min at 72°C. The PCR tubes were taken out in 3, 5, 7, 9, and 11 cycles, respectively, and kept in ice. Then, PCR products were assessed with 2 % agarose gel electrophoresis to determine the suitable number of PCR cycles (X). The suitable number of PCR cycles would provide the right PCR product and was confirmed by a brighter and smear-free band at 73 base pairs (Figure S3). Once the number of suitable PCR cycles (X) was determined, the bulk PCR reaction was run to generate 1 (or 2) mL of PCR mixture (20 µL of the 1st round PCR solution, 475 µL water, 500 µL of 2 × PCR solution, and 5 µL of Easy Taq Polymerase). The PCR

amplification conditions were set to 2 min at 95°C; X cycles of 45 s at 95°C; 30 s at 54°C; 30 s at 72°C; 2 min at 72°C.

After a bulk PCR, 20 µL of neutravidin beads were washed two times with 400 µL of SELEX buffer and incubated with 1 mL of PCR products for 15 min, rotating at RT. Then, the beads were washed twice with 400 µL of the SELEX buffer. The sense strand was separated from the beads by denaturing in 200 µL of 100 mM NaOH solution for 1 min; 0.2 M HCl immediately neutralized the solution. Then, the beads were again eluted by 212 µL of SELEX buffer, combined with the previous solution, and centrifuged using a desalting column (10K) at 12000 g for 10 min. The remaining solution in the desalting column was washed twice using 400 µL of the SELEX buffer. The eluted library was quantified by nanodrop. Then, it was treated at 95 °C for 5 min, ice for 5 min, and RT for 5 min and stored at -20°C. The 100 pmol DNA library (~2 µg) was incubated with 100 pmol protein (bead complex) for subsequent selection rounds. The amount of protein and incubation time were consistently decreased for the following selection rounds to increase the selection pressure (Table S1), while the number of washes to the PBL complex was consistently increased to ensure the removal of the unspecific libraries. The bound libraries were eluted twice by 30 µL of hot water at 95°C. Then, 20 µL from the total 60 µL elution was used for the PCR amplification, and the remaining 40 µL was stored at -20° C. The PCR amplification, purification, desalting, and quantification were similarly followed for the subsequent rounds as they were in the first SELEX. However, after the second SELEX, we introduced the counter selection (CS) in every other round. For that, we incubated the ssDNA with the unembellished Ni-NTA magnetic beads. The unspecific library bound to the magnetic beads was discarded, while the specific library in the supernatant was used to start the next selection round.

Specificity test by flow cytometry. A solution containing 200 nM of his-tagged RNAP protein complex was mixed with 100 µL of PBS-Mg (SELEX buffer) and 2 µL of Ni-NTA magnetic beads (Ref# 062N-A). The mixture was rotated for 1 hour at room temperature, with occasional tapping for mixing. Afterward, the bead/protein complex was washed twice using 100 µL of PBS-Mg. Next, 50 pmol of 6-FAM-labeled ssDNA was prepared in 100 µL of PBS-Mg. The ssDNA was subjected to heat treatment at 95 °C for 5 minutes, followed by cooling on ice (or at 4 °C) for 5 minutes, and then allowed to equilibrate at room temperature for an additional 5 minutes. The 500 nM 6-FAM-labeled ssDNA solution was incubated with 1 µL of 100 × tRNA and the bead/protein complex for 1 hour at room temperature, with continuous rotation and occasional tapping. Following incubation, the beads were washed twice and then resuspended in 80 µL of PBS-Mg. Finally, the bound fluorescence-labeled sequences were analyzed using flow cytometry (Catalog # 0500-4005; Guava easyCyte 5HT), which counted approximately 5,000 events. The average from three such measurements was used for analysis.

Sample preparation for Next-generation sequencing (NGS). The ssDNA pool of 11 SELEX rounds was prepared for NGS. The amplicon sequence library was prepared as mentioned in Figure S5. Two sets of primer sequences were designed, and PCR reactions were carried out. For the annealing process using the first set of primer sequences, the cycling conditions were as follows: 4 minutes at 95 °C, followed by 21 cycles of 45 seconds at 95 °C, 30 seconds at 58 °C, and 30 seconds at 72 °C. A final extension step was conducted for 4 minutes at 72 °C. The optimal number of cycles for the first NGS PCR, which is 21 cycles, was determined from a 2% agarose gel. This initial gel was run specifically to establish the optimal cycling conditions. After the first NGS PCR, the products were analyzed on an agarose gel, and purification was performed using the E.Z.N.A.® Gel Extraction Kit (Ref# D2500-01). In the second NGS PCR, the primers known as NGS-F2 and NGS-R2 included both the overhang regions and the adapter sequence, which are

crucial for properly attaching the oligonucleotides to the flow cell. The PCR reactions were conducted for 12 cycles, following these cycling conditions: 4 minutes at 95 °C, followed by 21 cycles of 45 seconds at 95 °C, 30 seconds at 58 °C, and 30 seconds at 72 °C, with a final extension step of 4 minutes at 72 °C. The optimal cycle number of 12 was determined based on the band brightness observed on a 2% agarose gel prior to conducting these 12 cycles. The PCR product was purified again to isolate the sample DNA using the E.Z.N.A.® Gel Extraction Kit (Ref# D2500-01) and subsequently sequenced by Azenta (Burlington, MA).

Flow cytometry experiment to measure the binding affinity of the aptamers: A total of 100 µL of 200 nM His-tagged RNAP protein was prepared in SELEX buffer and incubated with 1 µL of Ni-NTA magnetic beads (Ref# 062N-A; G-Biosciences) while rotating for 1 hour at room temperature (RT). After incubation, the Ni-NTA bead/RNAP protein complex was washed twice with 200 µL of SELEX buffer and then incubated with 100 µL of FAM-labeled aptamer at concentrations of 3, 10, 30, 100, 300, and 1000 nM, prepared in SELEX buffer, for 1 hour at RT with rotation. Following this, the beads were washed two times with 200 µL of SELEX buffer and finally resuspended in 100 µL of SELEX buffer. The FAM-labeled aptamers bound to the protein/bead complex were analyzed using flow cytometry (Catalog # 0500-4005; Guava easyCyte 5HT), counting approximately 5,000 events. Each experiment was conducted in triplicate to calculate the mean fluorescence intensity (XC) and standard error. The binding affinity (Kd) of the R2 aptamer against the RNAP protein was determined through an intensity versus concentration plot using Origin software.

Aptamer based protein capture. It was conducted using a multi-step protocol. Initially, an aptamer-biotin complex was formed by mixing an aptamer with a poly-A tail and biotin T-22 in a 1:1.2 molar ratio in SELEX buffer, achieving a final concentration of 500 nM. The mixture was heated at 95°C for 5 minutes and then cooled slowly to room temperature to facilitate proper annealing. Neutravidin beads (Thermo Scientific #REF 29204) were prepared by washing them three times with SELEX buffer and then resuspending them in a homogeneous slurry. The annealed aptamer-biotin complex was immobilized by incubating it with 20 µL of neutravidin beads for 30 minutes at room temperature while gently rotating the mixture. After immobilization, the beads were washed three times with binding buffer to eliminate any unbound aptamer. RNAP was added to the immobilized aptamer beads to capture proteins and incubated for 1 hour at room temperature with gentle rotation. To determine whether the R2 aptamer can capture the RNAP in the presence of other proteins, we also added 1 µg of BSA during the RNAP incubation. Nonspecifically bound proteins were washed away with three consecutive washes using SELEX buffer. The bound RNAP was then eluted by adding elution buffer to the beads and heating the mixture at 95°C for 5 minutes. The supernatant, containing pure RNAP, was collected and checked by SDS-PAGE to determine whether the R2 aptamer effectively pulled the RNAP.

In vitro transcription assay. A transcription volume of 20 µL was prepared, which included 12.1 µL of bio pure water, 2 µL of 10X reaction buffer, 1.4 µL of NTP from a 25 mM stock solution, 2 µg of template DNA (TGCACTTGACAAAGTCCTAAGGATCGATGTATAATCGCAGCGAGACGGTCGGGTCCAGATATTCGTATCTGTCGA GTAGAGTGTGGGCTC), 0.5 µL of RNase inhibitor, and 2 µ of RNA polymerase. Three tubes were prepared: one without the R2 aptamer and two with 0.1 µM and 1 µM concentrations of the R2 aptamer added. The tubes were incubated at 37°C for 24 hours. After incubation, urea PAGE gel electrophoresis was performed, and the data were subsequently analyzed by SYBR Green staining. The broccoli RNA aptamer (49 nt, GAGACGGUCGGGUCCAGAUAUUCGUAUCUGUCGAGUAGAGUGUGGGCUC) was used as a size control in the gel.

Supporting Figures:

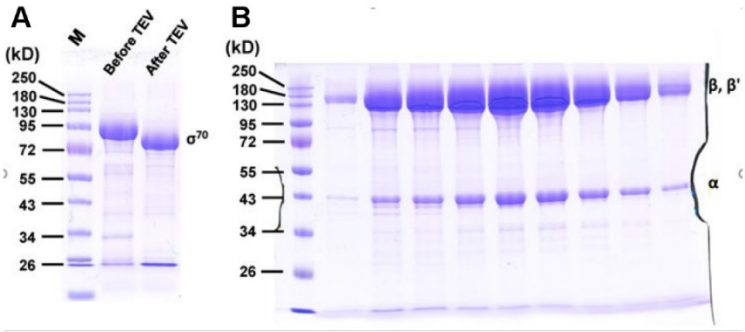

Figure S1. Purification of *P. aeruginosa* RNAP and RpoD (σ70). (A) Purified MBP-RpoD was cleaved by a TEV enzyme. (B) Harvested fractions of *P. aeruginosa* RNAP from Superdex 200 were displayed.

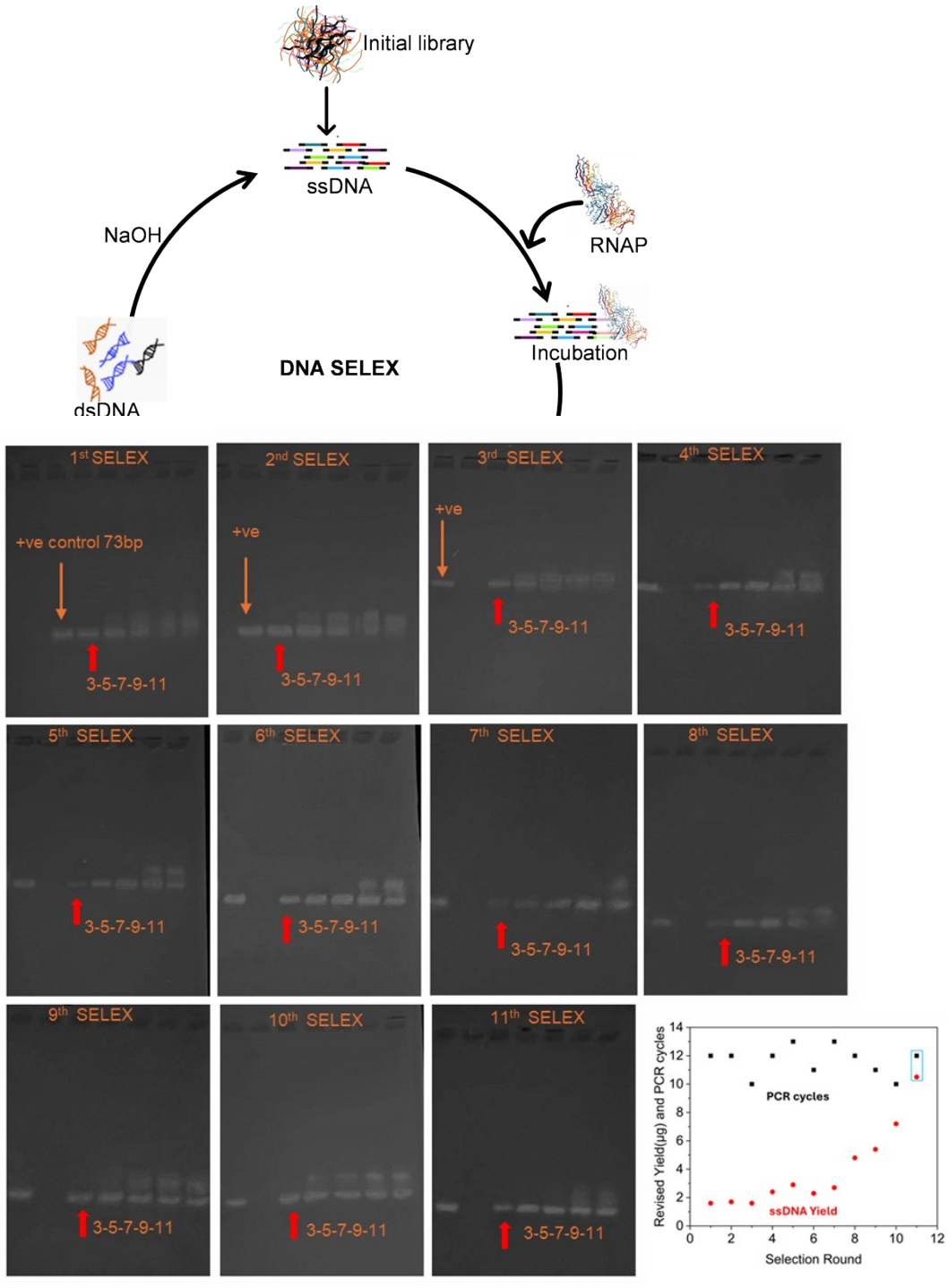

Figure S3. The gel electrophoresis data corresponds to the selection process of the R2 aptamer. The gel electrophoresis results evaluate the quality of the PCR product to identify the optimal number of PCR cycles (indicated by the upward red arrow) needed for DNA amplification. The correct size of the PCR product comprises 73 base pairs of nucleotides. A positive control (the accurate PCR product from the initial selection) was used to establish the sizes of the PCR products. Before optimization, rounds 1 and 2 underwent 9 PCR cycles, while rounds 3 through 11 completed 7 PCR cycles. The relationship between yield and PCR cycles over the selection rounds (located in the lower right corner) reveals that the 11th selection round demonstrates the highest level of enrichment.

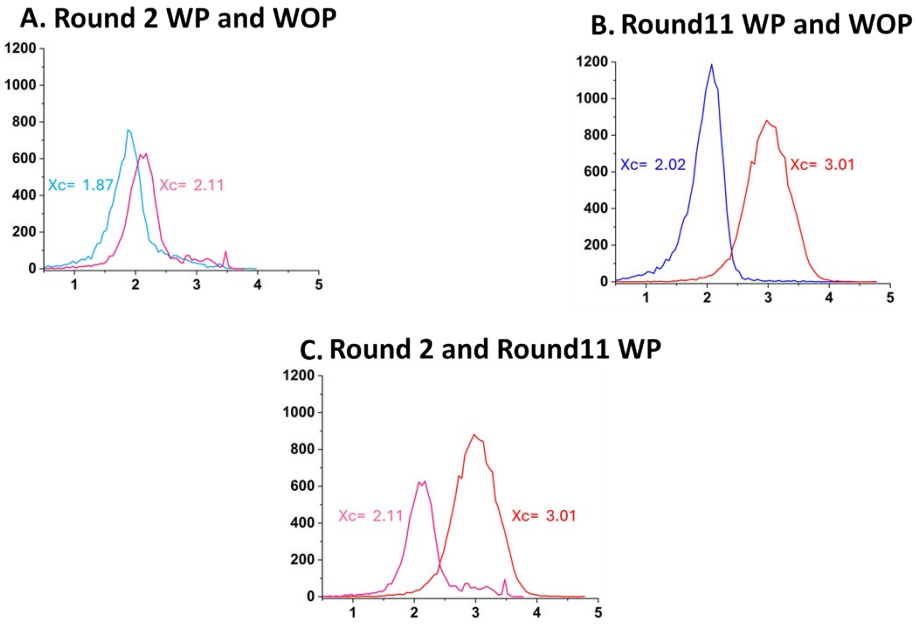

Figure S4. Specificity test for SELEX cycle rounds 2 and 11 using single-stranded DNA (ssDNA) from each round. The FAM-labeled ssDNA from rounds 2 and 11 was incubated with and without the protein to assess specificity. The results show a shift in mean fluorescence intensity, indicating that round 11 is more specific compared to the other experimental conditions. In figure S4 A the blue cyan is without the target and the graph in the pink is with the target. Similarly, in figure S4 B the dark blue color is without the target and the graph in red is with target protein. In figure S4 we plotted the with target only for round 2 and round 11 to see the shift and as clear from the graph the round 11 is highly specific than round 2 ssDNA with the target.

Figure S5. NGS amplicon workflow. A two-step PCR procedure was used to attach the sequencing primer, overhang region, and adapter sequences. The appropriate number of PCR cycles was determined through agarose gel electrophoresis to optimize the amplification process, allowing for the assessment of DNA product size and quantity. This step ensured that the amplification was efficient and adequate for subsequent sequencing analysis. The number of PCR cycle required was determined using the agarose gel.

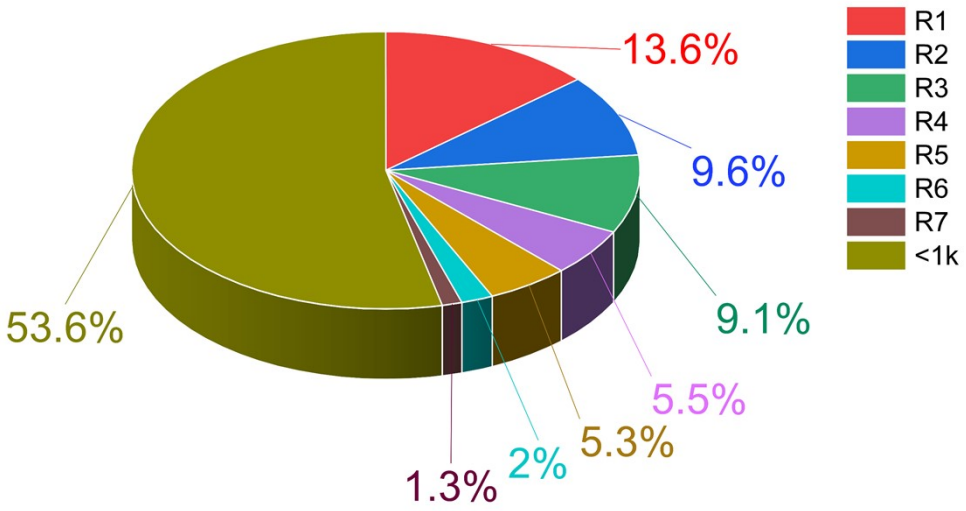

Figure S6. A three-dimensional pie chart depicting the frequency distribution of sequence repetition in the NGS dataset. The sequencing generated 101,725 reads. The three-dimensional pie chart displays the frequency of repeated sequences, with the top seven each exceeding 1,000 reads.

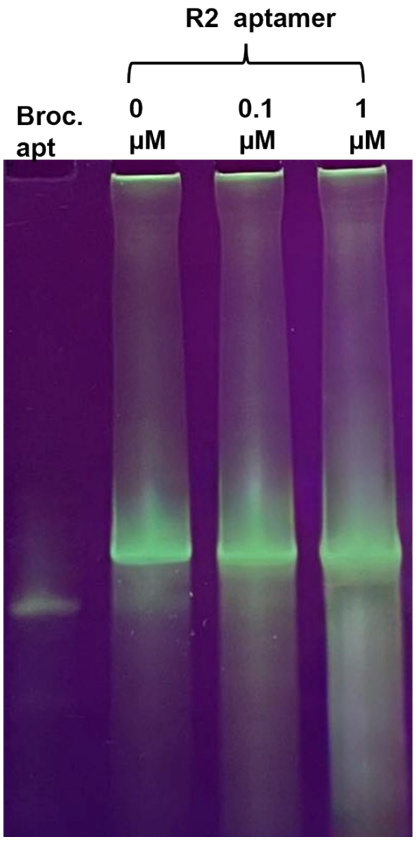

Figure S7. UREA PAGE analysis demonstrating the results of the transcription interference assay, evaluating the effect of the R2 aptamer. Lane 1 is the Broccoli RNA aptamer as a size control. Lane 2 is the RNAP based in vitro transcription without the aptamer R2. Lanes 3 and 4 are with 0.1 µM and 1 µM of the R2 aptamer in the transcription sample, respectively.

Table S1: The details of the selection cycles.

Round	Target protein (pmol)	Counter Selection*	Binding (time)	Wash repeat	Input Library (µg)	PCR cycles	ssDNA conc. (ng/µL)	Yield (µg)	Revised yield (µg)
1	100		1h	2	3 nmole	9+3=12	20.2	1.6	1.6
2	100		1h	2	1.6	9+3=12	14.8	1.7	1.7
3	100	2 µl, 5min	1h	3	1.7	7+3=10	16.3	1.6	1.6
4	50		1h	3	1.6	7+5=12	10.2	1.2	2.4
5	50	2 µl, 10 min	50 min	4	1.2	7+6=13	14.5	1.4	2.9
6	50		50 min	4	1.4	7+4=11	19.6	1.1	2.3
7	50	2.5µl, 10 min	50 min	5	1.1	7+6=13	15.0	1.3	2.7
8	33		40 min	5	1.3	7+5=12	13.5	1.6	4.8
9	33	2.5µl, 15 min	40 min	6	1.6	7+4=11	18.3	1.8	5.4
10	33		40 min	6	1.8	7+3=10	26.7	2.4	7.2
11	33	2.5µl, 20 min	30 min	7	2.4	7+5=12	43.9	3.5	10.5

Table S2: The sequences obtained from the NGS. The randomized sequences of the aptamers are flanked by reverse and forward primer on their 5' and 3' ends respectively as represented by 5'- CAAGGAGCGACCAGAGG-N40-TGGCATCCTTCAGCCC -3'

Aptamer	Sequences	Repetitions	Percentage(%)
R1	5'-CAAGGAGCGACCAGAGGTTTCACGTAAAGAGGAGGGAATACGCCGAGCATTTAAGAGTGGCATCCTTCAGCCC -3'	13858	13.6
R2	5'- CAAGGAGCGACCAGAGGGTAAACAGGAGAATAAGAAATTCAAGGATTTAAGACGATGTGGCATCCTTCAGCCC -3'	9774	9.6
R3	5'- CAAGGAGCGACCAGAGGCCATGCGATCTCAGGCGGGTGTCTGAACCTTTACTCCTCATGGCATCCTTCAGCCC -3'	9275	9.1
R4	5'- CAAGGAGCGACCAGAGGCACCATGTTTCGAGGATTCATAAGGTTATTAACAGGAGAATGGCATCCTTCAGCCC -3'	5623	5.5
R5	5'- CAAGGAGCGACCAGAGGCTATTAACAGGACTTCGGAGGATTCATTCCTATGGCGCGATGGCATCCTTCAGCCC -3'	5341	5.3
R6	5'- CAAGGAGCGACCAGAGGCCAGAGGATTCATCTATAGCAGATCTATTTATTAACAGGATGGCATCCTTCAGCCC -3'	2072	2