Supplementary Information

Enriching thermodynamically best aptamers by addressing the kinetic aspect of the DNA strand-displacement reaction

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Materials and Methods

Chemicals

The DNA samples used in this study were purchased from Integrated DNA Technologies (Coralville, IA, USA) (see Table S1 for sequences). Streptavidin agarose resin was obtained from Thermo Scientific (IL, USA), while metronidazole and 6-aminopenicillanic acid were sourced from Aladdin (Shanghai, China). Sodium chloride and magnesium chloride were purchased from Amerseco (Framingham, MA, USA). Ampicillin and penicillin G were obtained from Sigma-Aldrich (Burlington, MA, USA), and kanamycin A was supplied by Bio Basic Canada Inc (Markham, ON, Canada). Sodium hydroxide, aspirin, hydrochloric acid, voriconazole, and Amicon Ultra-0.5 centrifugal filter units (10 kDa molecular weight cutoff) were purchased from Millipore-Sigma (Oakville, ON, Canada). Micro bio-spin chromatography columns and SsoFast EvaGreen supermix were obtained from Bio-Rad (Hercules, CA, USA). dNTP mix, Taq DNA polymerase with ThermoPol buffer, and the low molecular weight DNA ladder were purchased from New England Biolabs (Ipswich, MA, USA). All buffers and solutions were prepared using Milli-Q water. The SELEX buffer for ampicillin consisted of 50 mM MES (pH 6.1), 500 mM NaCl, and 10 mM $MgCl_2$, while the SELEX buffer for adenosine contained 20 mM HEPES (pH 7.4), 150 mM NaCl, and 2 mM $MgCl_2$.

SELEX

The library design (Table S1) and selection method (Figure S1) were based on previous reports of the Stojanovic group[1] with some modifications. For each round, the single-stranded library was annealed by heating it with a threefold excess of biotinylated capture strand at 95°C for 3 min, followed by gradual cooling to room temperature in SELEX buffer. Meanwhile, 200 µL of agarose resin without streptavidin modification was introduced into a micro chromatography column and washed five times with 500 µL of SELEX buffer. Next, 20 µL of streptavidin agarose resin was added to the single-stranded library tube and incubated for 10 min. The resin, after incubation, was then transferred to the column containing the non-streptavidin-modified agarose resin. The column was subsequently washed 12 times to remove unbound library. After washing, 750 µL of target solution (dissolved in SELEX buffer) was added. For SELEX1, SELEX2, and ADESELEX1, the eluted DNA was directly collected using gravity flow. In contrast, for SELEX3, SELEX4, and ADESELEX2, the target was incubated in the micro chromatography column for 10 min before

being collected via gravity flow. The collected DNA was concentrated using a 10 kDa molecular weight cutoff filter and washed with Milli-Q water three to five times. The final volume of purified DNA was adjusted to 60 μL with Milli-Q water. A biotinylated reverse primer was used to synthesize 1 mL of PCR products, which were then incubated with 50 μL of streptavidin agarose resin for 20 min and washed ten times with a strand separation buffer (50 mM HEPES, pH 7.4, 150 mM NaCl). Subsequently, 600 μL of 0.2 M NaOH was added to the column and incubated for 10 min to elute the single-stranded DNA. After neutralization, the DNA was concentrated to 60 μL using a 10 kDa filter, and its concentration was measured with a Spark microplate reader (Tecan) before being used in the next round of selection. The target concentrations and incubation times for the various selections are summarized in Table S2.

Isothermal Titration Calorimetry (ITC)

The ITC experiments were conducted using MicroCal iTC200. Both aptamers and targets were dissolved in their own SELEX buffer and degassed by sonication for 5 min before the start of the experiment. The cell chamber was loaded with 205 μL aptamer solution, while the syringe contained 40.5 μL of target solution. All titration experiments were conducted at a temperature of 25°C. The obtained titration curves were fitted using a one-site binding model by Origin software.

ThT fluorescence spectroscopy

In the ThT binding assays, the aptamer and ThT were prepared at final concentrations of 500 nM and 10 μM, respectively, in their respective SELEX buffers, with a total volume of 500 μL. Fluorescence measurements were performed on a Horiba Fluoromax 4 fluorometer, with excitation at 420 nm and emission at 490 nm. The temperature was maintained at 25°C using a water bath, and titrations began once a stable background fluorescence was confirmed. [2] Each experiment was performed in triplicate. The K_d value was fitted using $y = y_0 + aK_d/(K_d + x)$, where x represents the concentration of the titrated target and a is the maximal signal change upon saturation.[3]

In the metal-dependent experiments, the Na^+ concentration was varied among 0 mM, 150 mM, and 500 mM. To assess the effect of Mg^{2+}, the system was supplemented with 0 mM, 2 mM, or 10 mM Mg^{2+}. In experiments with 0 mM $MgCl_2$, 1 mM EDTA was added to chelate any residual divalent cations present in the water. All solutions were prepared using Milli-Q water.

Table S1. The DNA sequence used in this work

DNA names	Sequences (from 5' to 3')
N30 library	GGAGGCTCTCGGGACGAC-N30-GTCGTCCCGATGCTCTGAATCACA
N36 library	GGAGGCTCTCGGGACGAC-N36-GTCGTCCCGATCACTTGAATGGTCT
Reverse primer (RP)	CTGTGATTCAGAGCATCGGGACG
Biot-Reverse primer	/5Biosg/ CTGTGATTCAGAGCATCGGGACG
Biot-Column	GTCGTCCCGAGAGCCATA /3BioTEG/
Forward primer (FP)	GGAGGCTCTCGGGACGAC
AMP-H1	GACGACGCCGTCTCGTACTCAGCCGTCATCTTACTAGTCGTC
AMP-L2	GACGACGCCATCCCGTACCCAGCCGTCATCTTACTAGTCGTC
AMP-L3	GACGACGCCATCCCGTACTCTGCCGTCATCTTACTAGTCGTC
A10G	GACGACGCCGTCCCGTACCCAGCCGTCATCTTACTAGTCGTC
C13T	GACGACGCCATCTCGTACCCAGCCGTCATCTTACTAGTCGTC
C19T	GACGACGCCATCCCGTACTCAGCCGTCATCTTACTAGTCGT
G12A	ACGACGTTTGCAATGAGAAACGTATGGTTTCGAAGGTCGT
FAMAMP6H-1	/56-FAM/ GGCTCTCGGGACGACGCCGTCTCGTACTCAGCCGTCATCTTACTAGTCGTCCCGA
FAMAMP6L-2	/56-FAM/ GGCTCTCGGGACGACGCCATCCCGTACCCAGCCGTCATCTTACTAGTCGTCCCGA
FAM-Ade1301	/56-FAM/ CTCTCGACGACGTTTGCGATGAGAAACGTATGGTTTCGAAGGTCGTC
FAM-classical aptamer	/56-FAM/ CTCTCGACGACTGGGGGAGTATTGCGGAGGAAGTCGT
Quencher-strand1	GTCGTCCCGAGAGCC/3IABkFQ/
Quencher-strand2	AGTCGTCGAGAG /3IABkFQ/

Note: /5Biosg/ is biotinylation at the 5′-end, /3BioTEG/ is biotinylation at the 3′-end, FAM is carboxyfluorescein, and /3IABkFQ/ is Iowa Black FQ (IDT) quencher at the 3′-end.

Table S2. List target concentrations and incubation time used for different selection

SELEX	[Target]	Total round of SELEX	Additional incubation time
Ampicillin SELEX 1	[Ampicillin] (5 mM)	15	0
Ampicillin SELEX 2	[Ampicillin] (100 μM)	16	0
Ampicillin SELEX 3	[Ampicillin] (5 mM)	15	10 min
Ampicillin SELEX 4	[Ampicillin] (100 μM)	16	10 min
Adenosine SELEX 1	[Adenosine] (100 μM)	11	0
Adenosine SELEX 2	[Adenosine] (100 μM)	11	10 min
Voriconazole SELEX 1	[Voriconazole] (200 μM)	17	0
Voriconazole SELEX 2	[Voriconazole] (200 μM)	17	10 min

Table S3. Aptamer release kinetics from a short cDNA strand.

Target	Aptamer	Concentration	K_d	ΔF at 2 min	ΔF at 10 min	Ref
Adenosine	Ade 1301b	20 μM	0.2 μM	90%	100%	[4]
Caffeine	Caff203	200 μM	2.2 μM	37%	100%	[5]
B-Estradiol	CN-Es1	5 μM	83 nM	67%	67%	[6]
B-Estradiol	CN-Es1	5 μM	30 nM	85%	85%	[6]
L-Lactate	Lac201	10 mM	0.43 mM	50%	50%	[7]
Ampicillin	AMP-H1	5 mM	12.7 μM	80%	80%	This work
Ampicillin	AMP-L2	5 mM	1.8 μM	70%	90%	This work
Uric acid	UA-Na-1	10 μM	1.2 μM	70%	70%	[8]
Citrate	CA1	40 μM	4.4 μM	46%	85%	[9]
Theophylline	Theo2201	5 μM	0.51 μM	81%	81%	[10]

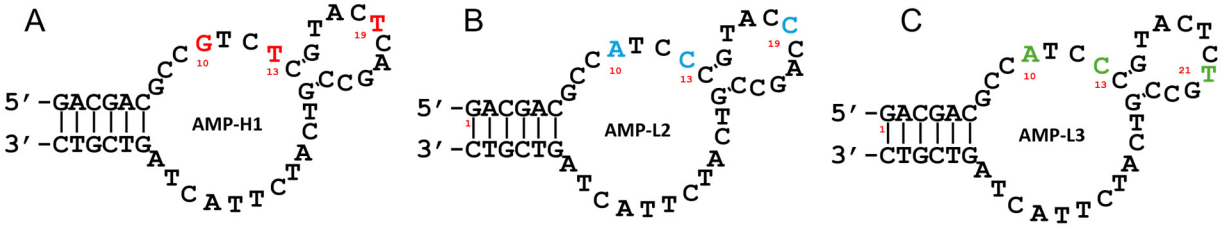

Figure S1. The secondary structure of the (A) AMP-H1, (B)AMP-L2, and (C) AMP-L3 aptamers generated by mFold. The differences are marked in colored nucleotides.

A

SELEX 3: 5 mM ampicillin with 10 min incubation

AMP-L2	GCCATCCCGTACCCAGCCGTCATCTTACTA	69.1%
AMP-H1	GCCGTCTCGTACTCAGCCGTCATCTTACTA	29.9%
DAMP-H3	GCCATCYCTTACCCAGCTKTCATCTTACTA	0.1%
DAMP-H4	GCCATCCCGTACCCAGCCGTCATCTTACTA	0.04%
DAMP-H5	GCCATCCCGTACCCAGCCGTCATCTTACTA	0.03%

B

SELEX 4: 100 µM ampicillin with 10 min incubation

AMP-L2	GCCATCCCGTACCCAGCCGTCATCTTACTA	93.2%
AMP-L3	GCCATCCCGTACTCTGCCGTCATCTTACTA	5.7%
AMP-H1	GCCGTCTCGTACTCAGCCGTCATCTTACTA	0.1%
DAMP-L4	CNARGCGYGRRWGAACTACTKCWGNCGASGT	0.01%
DAMP-L5	GCCAGCCAGGACCCAGCAGGGARATTACTA	0.01%

Figure S2. The ampicillin sequencing result for the two selections done with 10 min incubation (SELEX 3 and 4). Sequencing results for ampicillin selection showed a total yield of 38,591 sequences for the 5 mM ampicillin SELEX and 29,274 sequences for the 100 µM ampicillin SELEX.

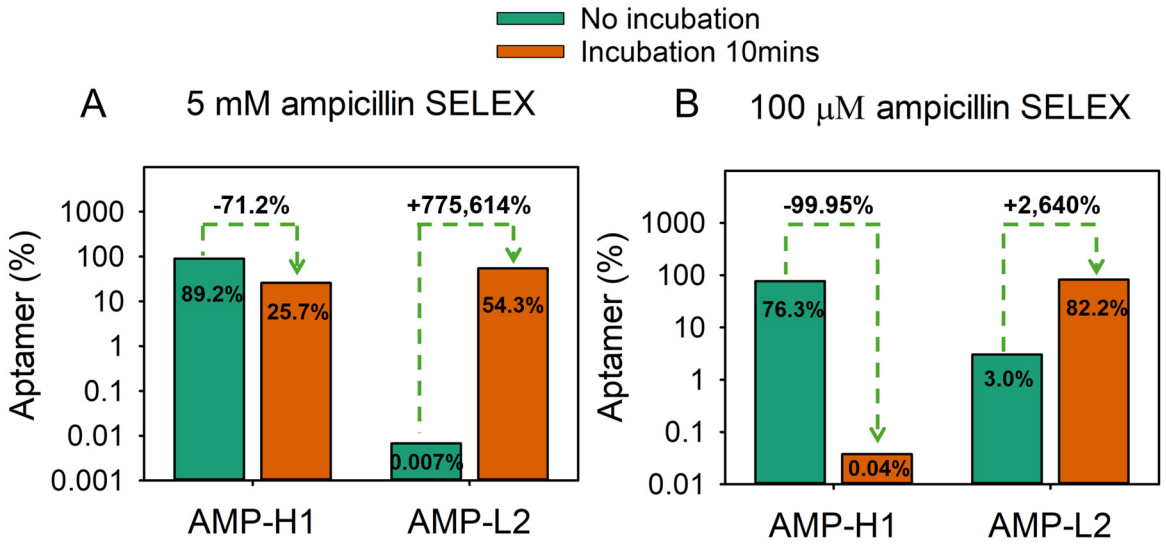

Figure S3. Distribution of analyzed sequences obtained from (A) 5 mM ampicillin SELEX and (B) 100 µM ampicillin SELEX performed under incubation target and without incubation.

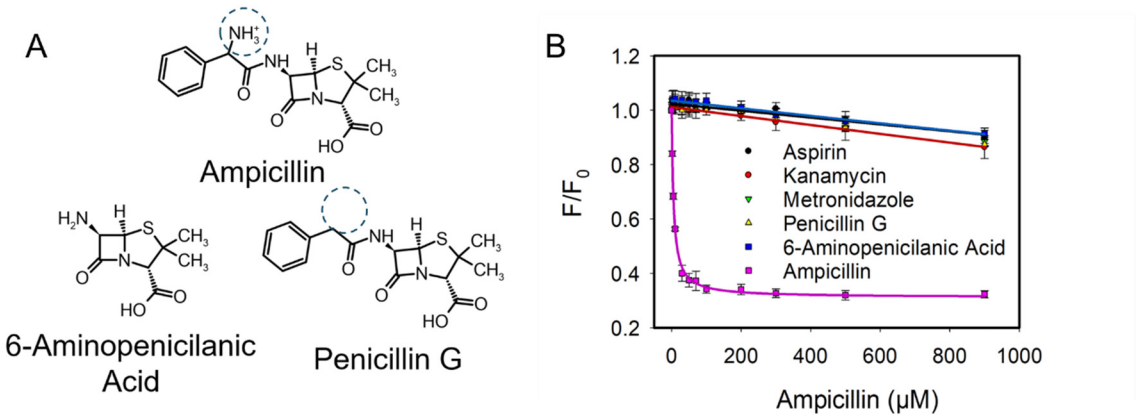

Figure S4. (A) Structures of some ampicillin analogs tested for selectivity. (B) The ThT fluorescence titration curve of the AMP-L2 aptamer for ampicillin analogs and other antibiotics and drugs.

A

ADESELEX 1: 100 μM adenosine No incubation

Family 1301

ADE1	GAAGGAGGACTCCGTAAGGGTACTCGTTTGCGATGAG	24.8%
ADE2	GAAGGAGGACTCCGTAAGGGTACTCGTTTGCAATGAG	11.0%
ADE5	GAAGAAACTCGGTATGGCGTAGTTTGTTTGCGATGAG	0.4%

Other family

ADE3	CGAGTTAGTGAGGCGGGGTTCTAGCTTCCA	1.8%
ADE4	GGCGAGGGGCTTGTCCGATGGCGATGAGTA	0.5%

B

ADESELEX 2: 100 μM adenosine with 10 min incubation

Family 1301

ADE1	GAAGGAGGACTCCGTAAGGGTACTCGTTTGCAATGAG	19.4%
ADE2	GAAGGAGGACTCCGTAAGGGTACTCGTTTGCGATGAG	7.4%
ADE3	GAAGAAACTCAGTATGGCATAGTTTGTTTGCGATGAG	1.0%
ADE4	GAAGAAACTCAGCATGGCATAGTTTGTTTGCGATGAG	0.6%
ADE5	GAAGAAACTCTGTACGGCATAGTTTGTTTGCGATGAG	0.3%

Figure S5. The adenosine sequence result (A) without incubation and (B) after incubation 10 min. Sequencing results for 100 μM adenosine selection showed a total yield of 40,966 sequences without incubation and 39,999 sequences with 10 min incubation.

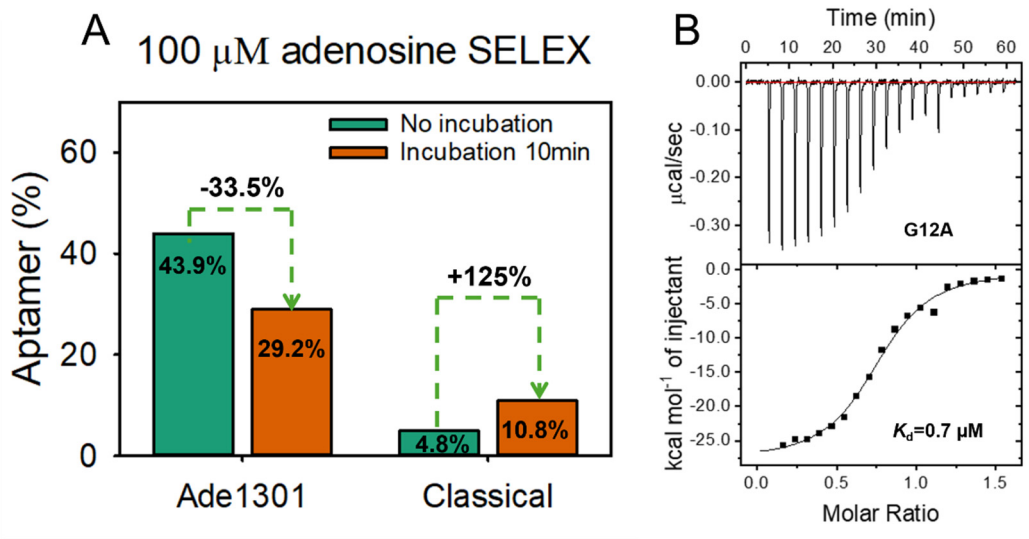

Figure S6. (A) Distribution of analyzed sequences obtained from 100 μM adenosine SELEX performed under two conditions: without additional incubation and with 10 min incubation. Two sequences were analyzed: Ade1301 along with the G12A mutant and the classical adenosine aptamer. (B) ITC traces and integrated heat profiles for titrating 150 μM adenosine to 20 μM G12A mutant of the Ade1301 aptamer.

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Family 1
vo4     GTAGCTGATCTAGTCTGCGAGCCATGGCTATGGGTA  2.6%
vo5     GTAGCGTAGTTTAGTCGCGAGTGCATAGATGCAGTA  2.4%
vo9     GTAGACAGGTTTACTTGGCGAGGCTTCCCAAGCGTA  1.5%
vo10    GTAGCAAACTGTGGTATGCGAGCCGTTACCCGGGTA  1.4%
vo12    ACGTAGCTGTTTAACTTGCGAGCACTTGTTGGTAGA  1.3%
vo13    GTAGCGATTTTCGCGAGTGGACATAAGGGGCCAGTA  1.3%
vo14    GTAGCTCGGTATGAGCGAGTACAGTTGTCTGTAGTA  1.3%
vo15    GCGTAGCCTTAGCGAGCCACGGTTTACGGGGGTAGA  1.2%
vo16    GTAGCACACTGCTGCGAGCTGTCTTTTCAACAGGTA  1.2%
vo17    GTAGCAGGTTACGGCTGCGAGCTCCTTGCGGAGGTA  1.1%
vo18    GTAGCAATGTTCTGCGAGTTTACGTTTCGTAAAGTA  1.1%
vo19    GTAGCACAATTTGCGAGCCCGGGATTTGCAGGGGTA  1.1%
vo20    GTAGGAATGGGTTCCGAGTCCAAGTACACTGGAGTA  1.1%
vo8     GTAGCAGGTTATCTGCGAGCGAGTTGAATCGAGATA  1.6%
vo6     GAGTCGGACATGGTATGCTCCGAGTAGGGCACCGTA  2.1%
vo7     GAGTAGGATTGTTTATCGTCCGTAGTTTTGCGAGGA  1.8%
vo11    GTAGGCATACACTTCGTATGGCGTAGCGTCAGCGAG  1.4%
vo2     GGTAGTGAATGGTTTATCTGAACGTAGCTAGCGAGC  3.6%
Ungrouped sequences
vo1      TGGGCGTAGTTGTGCGACGAATAAGTTCGTAGGCCT 4.3%
vo3      TTAAAGGGTTTGTTCCTTGTACCAGGAATGGATTTA 2.8%
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Figure S7. Alignment of the top 20 sequences from the voriconazole SELEX. 18 of them can be classified into one family feature with three groups of conserved trinucleotides separated by stem loops. The underlined nucleotides can base pair.

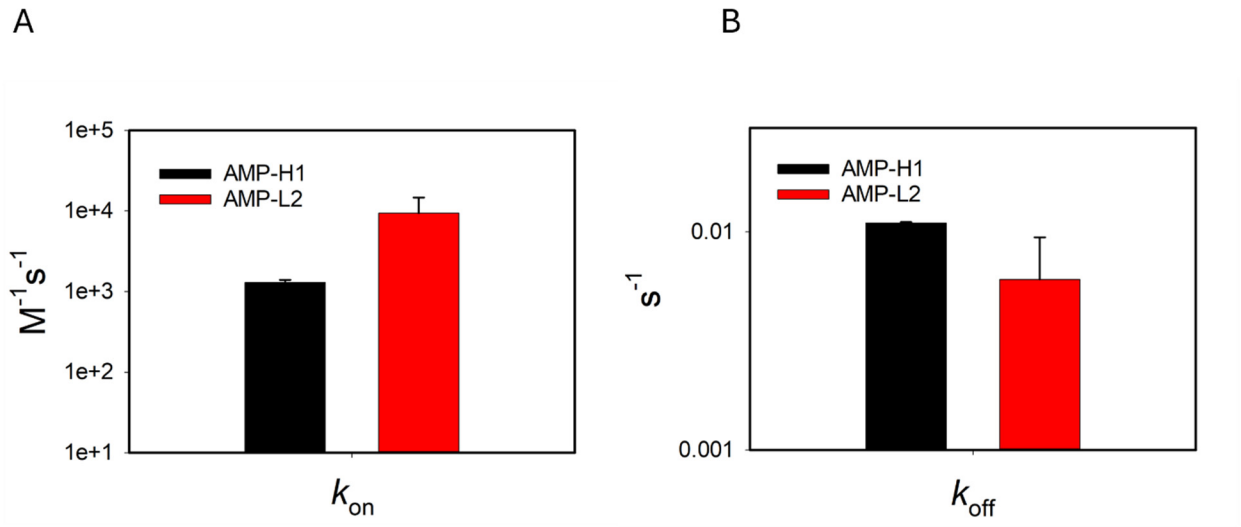

Figure S8. The (A) k_{on} and (B) k_{off} values of the two ampicillin aptamers from Affinimeter software using the Kinetic ITC method.

References

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