Supporting information for the article:

Isolation of Natural DNA Aptamers for Challenging Small-Molecule Targets – Cannabinoids

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ABSTRACT

Aptamers are nucleic acid-based affinity reagents that are isolated via an *in vitro* process known as systematic evolution of ligands by exponential enrichment (SELEX). Despite their great potential for a wide range of applications, there are relatively few high quality small-molecule binding aptamers, especially for 'challenging' targets that have low water solubility and/or limited moieties for aptamer recognition. The use of libraries containing chemically modified bases may improve the outcome of some SELEX experiments, but this approach is costly and yields inconsistent results. Here, we demonstrate that a thoughtfully-designed SELEX procedure with natural DNA libraries can isolate aptamers with high affinity and specificity for challenging small molecules, including targets for which such selections have previously failed. We first isolate a DNA aptamer with nanomolar affinity and high specificity for (-)-trans-Δ^9-tetrahydrocannabinol (THC), a target previously thought to be unsuitable for SELEX with natural DNA libraries. We subsequently isolated aptamers that exhibit strong affinity and cross-reactivity to two other highly challenging targets, the synthetic cannabinoids UR-144 and XLR-11, while maintaining excellent specificity against a wide range of non-target interferents. Our findings demonstrate that natural nucleic acid libraries may yield high quality aptamers for small-molecule targets, and we outline a robust workflow for isolating other such aptamers in future selection efforts.

Table of Contents

EXPERIMENTAL SECTION

Materials. Cannabinoids and their metabolites, including (−)-trans-Δ⁹-tetrahydrocannabinol (THC), (−)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH), (±)-11-hydroxy-THC (THC-OH), cannabinol (CBN), tetrahydrocannabivarin (THCV), tetrahydrocannabinolic acid (THCA), cannabidiol (CBD), cannabidiolic acid (CBDA), cannabigerol (CBG), cannabigerolic acid (CBGA), UR-144, XLR-11, UR-144 pentanoic acid metabolite (UR-144M), pentylone HCl, methcathinone HCl, α-pyrrolidinopentiophenone HCl, heroin HCl, and fentanyl HCl were purchased from Cayman Chemicals. Acetaminophen, alpha-tocopherol, albendazole, (±)-amphetamine hemisulfate, caffeine, clonazepam, cocaine HCl, granisetron, ibuprofen, (+)-methamphetamine HCl, nicotine, procaine HCl, (+)-pseudoephedrine HCl, serotonin HCl, tryptophan, 3-di(3-sulfopropyl)-4,5,4,5-dibenzo-9-ethylthiacarbocyanine triethylammonium salt (ETC) and all other chemicals were purchased from Sigma-Aldrich unless otherwise noted. Tween 20, formamide, SYBR Gold, streptavidin-coated agarose resin (capacity: 1-3 mg biotinylated bovine serum albumin (BSA)/ml resin), TOPO TA cloning kit, and ExoSAP-IT Express PCR Purification Kit were purchased from Thermo Fisher Scientific.

Peppermint (Starwest Botanicals), damiana (herbandflame.net), lemon balm (Organic Bio Herbs, Ltd.), and thyme (Straight from France) were purchased as dried leaves from online vendors. Each leaf extract was prepared as follows: 500 mg of leaves were mixed with 10 mL methanol, sonicated for 30 min at room temperature, and then left at 4°C overnight for extraction. The mixture was then filtered with a 0.22-µm syringe filter (Millipore) to remove insoluble contents. The final extracts (50 mg/mL) all showed a greenish-yellow color, and were stored at 4°C.

High-throughput sequencing. The round 16 and 17 pools from our synthetic cannabinoid SELEX experiment were prepared for sequencing at the FIU DNA Core Facility using an Ion Torrent Personal Genome Machine with an Ion 318 v2 chip (Thermo Fisher Scientific). The primer region of each sequence was trimmed by cutadapt,[1] and the population of sequences from each pool were characterized using FASTAptamer.[2] Clustering was also performed by FASTAptamer, where the XA1 and XA2 families comprised sequences that differ by no more than 6 nt (≥ 80% similarity) relative to their consensus.

Gel elution assay. The gel elution assay was performed as previously described.[3] 50 pmol of the SELEX pool was incubated with 250 pmol of biotinylated cDNA in 125 µL of selection buffer, heated at 95 °C for 10 min and cooled at room temperature over 20 min to form cDNA-library complexes. Afterward, a microcentrifugation column (Bio-Rad) was prepared by adding 125 µL of streptavidin-coated agarose beads and then washing with 250 µL of selection buffer five times. The cDNA-library complex was then added to the column and immobilized onto the beads, and the eluent was collected and flowed through the column twice more. The library-immobilized agarose beads were transferred into a microcentrifugation tube and washed five times by adding 625 µL of selection buffer, incubating on an end-over-end rotator for 5 min, followed by

centrifugation and removal of the supernatant. The volume of the library-immobilized bead solution was adjusted to 150 μL with selection buffer and aliquoted into seven tubes (20 μL/tube). Afterward, 50 μL of various concentrations of target (0, 10, 20, 50, 100, 250 or 500 μM) was added into each tube. After rotating for 60 min on an end-over-end rotator at room temperature, the beads were settled using a mini-centrifuge, and 40 μL of the supernatant containing the target-eluted strands was collected and set aside. The leftover solution (30 μL) was mixed with 50 μL of 98% formamide solution containing 10 mM EDTA and incubated at 90 °C for 10 min to completely release all DNA strands from the beads. The resulting solution contained both leftover target-eluted strands and non-target-eluted strands. We analyzed the target-eluted aptamer solution and formamide-treated library solution via 15% denaturing polyacrylamide gel electrophoresis (PAGE) and determined the concentrations of the strands based on standardized concentrations of ladder loaded in the gel. The elution percentage was calculated using the equation:

$$\theta = \frac{V1 \times Cs}{V2 \times Cs + V3 \times Cb} \times 100\% \tag{S1}$$

where θ is the fraction of target-eluted strands, Cs is the concentration of target-eluted strands in the supernatant, Cb is the concentration of strands in the formamide solution, $V1$ is the volume of solution before supernatant collection (estimated as 62 μL, with ~8 μL occupied by agarose beads), $V2$ is the volume of the collected supernatant containing target-eluted strands (40 μL), and $V3$ is the volume of solution after addition of formamide (80 μL). A calibration curve was created by plotting the fraction of eluted strands against the employed target concentration. The resulting curve was fitted with the Langmuir equation to determine the dissociation constant (K_D) of the enriched pool. The same protocol was used to determine the target cross-reactivity of the enriched pool for UR-144. The cross-reactivity was calculated using following equation:

$$Cross-reactivity\ (\%) = \frac{Target\ elution\ of\ analyte - Buffer\ elution}{Target\ elution\ of\ XLR-11 - Buffer\ elution} \times 100\% \tag{S2}$$

Isothermal titration calorimetry (ITC). ITC experiments were performed using a MicroCal iTC200 Instrument (Malvern) at 23 °C in the following buffer: 20 mM Tris-HCl, 20 mM NaCl, 0.5 mM $MgCl_2$, 5% DMSO, pH 7.4. For ITC experiments with THC1.2, various concentrations of THC1.2 in 64 μL reaction buffer were heated at 95 °C for 10 minutes and immediately cooled down on ice for 5 minutes. The aptamer solution was then loaded in the instrument syringe. The cell was loaded with 300 μL of THC, THC-OH, or THC-COOH solution in reaction buffer. For ITC experiments with XA1 and XA2, various concentrations of these aptamers were prepared in 300 μL reaction buffer and heated at 95 °C for 10 minutes and immediately cooled on ice for 5 minutes. The aptamer solution was then loaded in the cell. The target solution containing UR-144 or XLR-11 was then loaded in the instrument syringe. Each ITC experiment consisted of an initial purge injection of 0.4 μL and 19 successive injections of 2 μL, with 300 sec spacing between every injection. The heat from each injection was integrated using the MicroCal analysis kit in the Origin 7 software, corrected with dilution heat obtained from analyte-to-buffer titrations, and fitted with a single-site binding model to calculate K_D. Experimental conditions and binding parameters for each titration are listed in **Table S5**.

Fluorophore-quencher assay. The fluorophore-quencher assay was performed as previously,[4] with some modifications. First, the affinity of F-THC1.2 and F-XA1 for Q-cDNA was characterized. F-THC1.2 or F-XA1 (final concentration 40 nM) and different concentrations of Q-cDNA (final concentration 0, 3.1, 6.3, 12.5, 25, 37.5, 50, 75, 125, or 150 nM for F-THC1.2; 0, 6.3, 12.5, 25, 500, 75, 100, 150, 225, or 300 nM for F-XA1) were mixed in 80 µL reaction buffer (20 mM Tris-HCl, 20 mM NaCl, 0.5 mM $MgCl_2$, 5% DMSO, pH 7.4). The samples were then heated at 95 °C for 5 min and cooled to 25 °C at a rate of 0.1°C/s on a Bio-Rad C1000 thermal cycler. After an additional 5 min at room temperature, 75 µL of each sample was loaded into a 96-well plate, and the fluorescence intensity of each sample (λ_{ex} = 648 nm, λ_{em} = 668 nm) was recorded using a Tecan M1000 Pro plate reader. The concentration of unbound aptamer for each sample was calculated as $F/F_0 \times 50$ nM, where F and F_0 are the fluorescence of the sample in the presence and absence of Q-cDNA, respectively. The free Q-cDNA concentrations for each sample were then calculated as $C_Q - F/F_0 \times 50$ nM, where C_Q is the total concentration of Q-cDNA added to each sample. The fluorescence of each sample was plotted against the free concentration of Q-cDNA. The dissociation constant between F-THC1.2 and Q-cDNA (K_{D1}) was determined based on the concentration of free Q-cDNA where half of the fluorescence was quenched.

A second experiment was used to quantify the affinity of the target for the aptamer-cDNA complex. F-THC1.2 or F-XA1 (final concentration 40 nM) and Q-cDNA (final concentration 125 nM for F-THC1.2 and 300 nM for F-XA1) were mixed in reaction buffer, and the solution was heated and cooled as described above. Different concentrations of analyte (THC, THC-OH or THC-COOH for F-THC1.2; UR-144 or XLR-11 for F-XA1) were then mixed with the aptamer-cDNA complexes and incubated for 30 min at room temperature. The samples were then loaded into a 96-well plate (total volume 75 µL), and the fluorescence of each sample at 668 nm (excitation at 648 nm) was recorded and plotted against the target concentration. These results were fitted with the dose-response curve to determine the EC_{50} concentrations, at which half of the fluorescence was recovered. K_{D2} for the target and aptamer-cDNA complex was calculated as C_{50}/EC_{50}, where C_{50} is the free Q-cDNA concentration at which EC_{50} is reached. Finally, the K_D of the free aptamers for their targets was calculated based on K_{D1}/K_{D2}.

For fluorescence-based sensing of analytes, sensor performance was assessed using signal gain. The signal gain of each sample was calculated as $(F-F_0)/F_0 \times 100\%$, where and F_0 and F respectively represent the fluorescence at 668 nm in the absence or presence of target. Each experiment was performed three times. The limit of detection of the assay was determined as the lowest analyte concentration that yielded an average signal greater than three times the standard deviation. For characterization of aptamer specificity, aptamer-cDNA mixtures were prepared as described above. 10 or 50 µM of different analytes were then mixed with the aptamer-cDNA complexes and incubated for 30 min at room temperature. The samples were then loaded into a 96-well plate (total volume 75 µL), and the fluorescence spectra were recorded at room

temperature. The signal gain of each sample was calculated as described above. The cross-reactivity of each analyte was calculated based on their signal gains, where the signal gain from 10 µM THC and UR-144 was defined as 100% cross-reactivity for F-THC1.2 and F-XA1, respectively.

Dye displacement assay for detection of THC. The assay was performed at room temperature. For THC detection, 3.1 µL of 142 µM THC1.2 was incubated in 51.3 µL Tris–HCl buffer (pH 7.4) (final concentration 10 mM) for 5 min. Then, 0.8 µL of 1% Triton-X100 (w/v) and 0.8 µL of 400 µM ETC (dissolved in 100% DMSO) was added to the aptamer solution and incubated for 1 min. Subsequently, 16 µL of salt solution (final concentration 20 mM NaCl, 0.5 mM $MgCl_2$) was added. Immediately afterwards, 8 µL of 55% DMSO or varying concentrations of Δ^9-tetrahydrocannabinol (THC) dissolved in 55% DMSO (final concentrations: 0.25, 0.5, 1, 1.5, 2, 2.5, 3.5, 5, 10, or 20 µM) were added to the reaction mixture, and 75 µL of the resulting solution was loaded into a transparent 384-well plate. UV-vis spectra were recorded from 400–800 nm using a Tecan Infinite M1000 Pro. Area under the curve was calculated using Origin software for the dye monomer (Area 1, 500–620 nm) and dye aggregate (Area 2, 620–680 nm). Signal gain was calculated using $(R - R_0)/R_0$, where R and R_0 is the ratio Area 2/Area 1 with and without THC, respectively. To determine the cross reactivity of our assay, the same procedure was performed for 5 µM THC, THC-COOH, THCV, or CBN; 25 µM THCA, CBD, CBDA, CBG, CBGA, UR-144, or XLR-11; and 100 µM cocaine, amphetamine, heroin, α-pyrrolidinopentiophenone (α-PVP), methamphetamine, pentylone, procaine, acetaminophen, ibuprofen, nicotine, caffeine, clonazepam, or fentanyl. Cross-reactivity was calculated relative to the signal gain produced by 5 µM THC.

Table S1. DNA sequences used in this work.

Seq. ID	Sequence (5' to 3')
cDNA	TTTTTGTCGTAAGTTCTGCCATTTT/3STGBio/
Library	CGAGCATAGGCAGAACTTACGAC(N30)GTCGTAAGAGCGAGTCATTC
THC1.2	CTTACGACCCAGGGGGGTGGACAGGCGGGGGTTAGGGGGGTCGTAAG
XA1	CTTACGACTGTGGTCGGGTGGTGGGCCTCTAGAGGGGTGTCGTAAG
XA2	CTTACGACTGCGGGCATTTGTGGGGGGCGTCGGTGGGCGTCGTAAG
F-THC1.2	/5Cy5/GGCAGAACTTACGACCCAGGGGGGTGGACAGGCGGGGGTTAGGG GGGTCGTAAG
F-XA1	/5Cy5/GGCAGAACTTACGACTGTGGTCGGGTGGTGGGCCTCTAGAGGGGT GTCGTAAG
Q-cDNA	GTCGTAAGTTCTGC/3IAbRQsp/

/3STGBio/ = biotin modification; /5Cy5/ = Cy5 modification; /3IAbRQsp/ = Iowa Black RQ quencher modification.

Table S2. SELEX conditions for isolating a THC-binding aptamer.

Round #	Pool size (pmole)	Counter-targets				Targets (nmole)
1	1000	N/A				150
2	423	3 mg of each leaf extract (peppermint, damiana, lemon balm, or thyme)				150
3	450	6 mg of each leaf extract		225 nmole COC, PRC, PSE and ACM, 75 nmole IBU	225 nmole AMP, PTL, MCA, and α-PVP, 75 nmole CLZ	150
4	300	10 mg of each leaf extract		450 nmole COC, PRC, PSE and ACM, 150 nmole IBU	450 nmole AMP, PTL, MCA, and α-PVP, 150 nmole CLZ	150
5	300					150
6	300					150
7	300	10 mg of each leaf extract		750 nmole, PRC, PSE and ACM, 250 nmole IBU	750 nmole AMP, PTL, MCA, and α-PVP, 250 nmole CLZ	150
8	150					150
9	150					75
10	150					75
11	150	10 mg of each leaf extract	1.25 mL 50% saliva 1.25 mL 50% urine	750 nmole, PRC, PSE and ACM, 250 nmole IBU	750 nmole AMP, PTL, MCA, and α-PVP, 250 nmole CLZ	75

cocaine (COC), procaine (PRC), pseudoephedrine (PSE), acetaminophen (ACM), ibuprofen (IBU), amphetamine (AMP), pentylone (PTL), methcathinone (MCA), α-pyrrolidinopentiophenone (α-PVP), clonazepam (CLZ)

Table S3. Experimental conditions for our three UR-144 and XLR-11 SELEX experiments.

Approach #	Round #	Tween 20	Pool size (pmole)	Counter-targets		Incubation	Target
				Matrices	Interferents		
1	1.1	0.05%	1000	N/A	N/A	N/A	375 nmole XLR-11 375 nmole UR-144
	2.1		500	1.25 mg each of peppermint, damiana, lemon balm, and thyme	225 nmole 5-HT, Trp, ABZ, TCP, THC, α-PVP, GNS, NIC, CAF, MET, and COC		375 nmole XLR-11 375 nmole UR-144
	3.1		500				225 nmole XLR-11 225 nmole UR-144
	4.1		400	3.75 mg each of peppermint, damiana, lemon balm, and thyme extract	225 nmole ABZ, TCP, and THC; 375 nmole 5-HT, Trp, α-PVP, GNS, NIC, CAF, MET, and COC		150 nmole XLR-11 150 nmole UR-144
	5.1		400				225 nmole XLR-11 225 nmole UR-144
	6.1		300				150 nmole XLR-11 150 nmole UR-144
2	1.2	N/A	1000	N/A	N/A	N/A	300 nmole XLR-11 300 nmole UR-144
	2.2		600	1.25 mg of each of peppermint, damiana, lemon balm, and thyme extract	75 nmole 5-HT, Trp, ABZ, TCP, THC, α-PVP, GNS, NIC, CAF, MET, and COC		225 nmole XLR-11 225 nmole UR-144
	3.2		500	3.75 mg of peppermint, damiana, lemon balm, and thyme extract	225 nmole 5-HT, Trp, ABZ, TCP, THC, α-PVP, GNS, NIC, CAF, MET, and COC		225 nmole XLR-11 225 nmole UR-144
	4.2		400		225 nmole ABZ, TCP, and THC; 375 nmole 5-HT, Trp, α-PVP, GNS, NIC, CAF, MET, and COC		150 nmole XLR-11 150 nmole UR-144
	5.2		250	7.5 mg each of peppermint, damiana, lemon balm, and thyme extract	225 nmole ABZ, TCP, and THC; 375 nmole 5-HT, Trp, α-PVP, GNS, NIC, CAF, and MET; 750 nmole COC		150 nmole UR-144
	6.2		250				150 nmole XLR-11
	7.2		200	7.5 mg each of damiana, lemon balm, and thyme extract; 10 mg peppermint extract	225 nmole ABZ; 375 nmole 5-HT, Trp, ABZ, TCP, THC, α-PVP, GNS, NIC, CAF, and MET; 750 nmole COC	10 min	75 nmole UR-144
	8.2		200				75 nmole XLR-11
3	5.3	N/A	250	7.5 mg of lemon balm and thyme extract; 10 mg each of peppermint and damiana extract	225 nmole ABZ; 375 nmole 5-HT, Trp, TCP, α-PVP, GNS, NIC, CAF, and MET; 600 nmole THC; 750 nmole COC	10 min	150 nmole UR-144
	6.3		250				150 nmole XLR-11
	7.3		200		225 nmole ABZ; 375 nmole 5-HT, Trp, TCP, α-PVP, GNS, NIC, CAF, and MET; 750 nmole COC; 1200 nmole THC		75 nmole UR-144
	8.3		200				75 nmole XLR-11
	9.3		150				150 nmole XLR-11
	10.3		150				150 nmole XLR-11
	11.3		150				150 nmole XLR-11
	12.3		150		225 nmole ABZ; 375 nmole 5-HT, Trp, TCP, α-PVP, GNS, NIC, CAF, and MET; 1250 nmole COC		75 nmole XLR-11
	13.3		150				37.5 nmole XLR-11
	14.3		150				37.5 nmole XLR-11
	15.3		150				75 nmole XLR-11
	16.3		150				75 nmole XLR-11
	17.3		100				37.5 nmole XLR-11

Serotonin (5-HT), tryptophan (Trp), albendazole (ABZ), α-tocopherol (TCP), tetrahydrocannabinol (THC), α-pyrrolidinopentiophenone (α-PVP), granisetron (GNS), nicotine (NIC), caffeine (CAF), cocaine (COC), methamphetamine (MET)

Table S4. Sequences of the random region and counts for clones from the round 11 THC SELEX pool.

No.	Sequences (5'-3')	Counts (total 44)
1	CCAGGGGGGTGGACAGGCGGGGGGTTAGGGGG	30
2	CCAGGGGGGTGGACAGACGGGGGGTTAGGGGG	1
3	CCAGGGGGGTGGACAGGCGGGGGGTCAGGGGG	1
4	CATGCCGACACCTTCAGAAGGTTCAGCGGA	1
5	CGGGCTGTCAACTGGGGAGTGCGGACTGGT	1
6	AACTGAGGCGGACAGAAGGGATCCGCGTGGT	1
7	GCGGCTGGAGCGTTAGGTCTCAAGGATAGG	1
8	GAGGAGACCTACCATTGTGACGGTAACGTT	1
9	CACCAGTGAGCCTCCCGGGATCGTGAAATG	1
10	ACGGATAACCCACGTGTATAGGTTGGAGTG	1
11	AGGGTAGAGCCTGATCAAGTGGTGAATTCT	1
12	GGCCTGTGTGCGGTCATTGACGCTGGCGCT	1
13	TGCGTACGTAAACTGTTTTGTTTGACCGTA	1
14	CGGATCAGCATACGTTGCGTAGTCCAACTG	1
15	GTTCGGTAGAGCTAGAATTGTGGCGGTGAC	1

Table S5. Experimental parameters for ITC experiments.

Experiment No.	Cell	Syringe	K_D (nM)
1	(−)-THC / 20 µM	THC1.2 / 150 µM	61 ± 25
2	(±)-THC-OH / 25 µM*	THC1.2 / 150 µM	556 ± 64
3	(−)-THC-COOH / 30 µM	THC1.2 / 150 µM	180 ± 36
4	XA1 /20 µM	XLR-11 / 200 µM	310 ± 70
5	XA1 /20 µM	UR-144 / 150 µM	127 ± 32
6	XA2 /20 µM	XLR-11 / 200 µM	394 ± 93
7	XA2 /20 µM	UR-144 / 150 µM	170 ± 44

*For ITC fitting, concentration was assumed to be 12.5 µM since it was a racemic mixture.

Table S6. Experimental results of ITC.

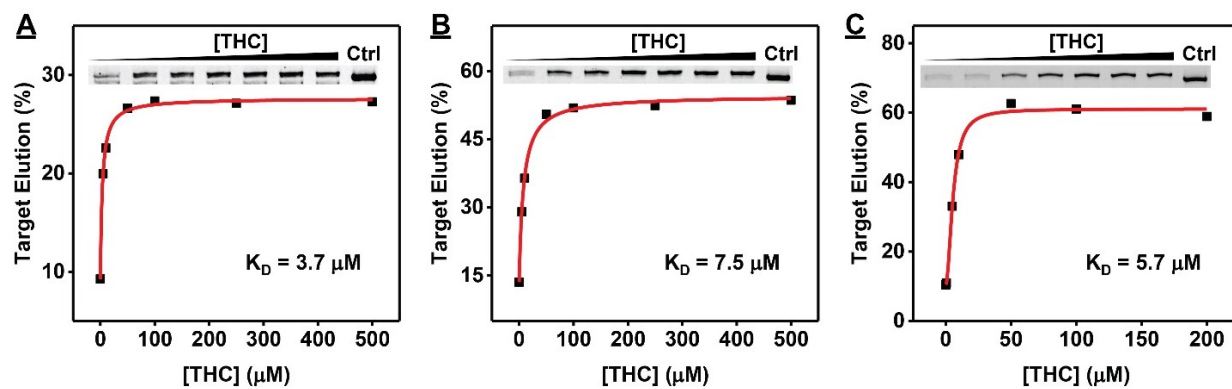| Aptamer ID	Ligand	K_D (nM)	ΔH (kcal/mol)	ΔS (cal/mol·K)
THC1.2	(−)-THC	61 ± 25	-12.7 ± 0.3	-9.8
THC1.2	(−)-THC-COOH	556 ± 64	-12.7 ± 0.2	-12.0
THC1.2	(±)-THC-OH	180 ± 36	-14.9 ± 0.5	-21.6
XA1	XLR-11	310 ± 70	-11.9 ± 0.3	-10.5
XA1	UR-144	127 ± 32	-11.9 ± 0.3	-8.8
XA2	XLR-11	394 ± 93	-5.3 ± 0.1	11.5
XA2	UR-144	170 ± 44	-6.9 ± 0.2	7.56

Figure S1. THC affinity of the pools from round (**A**) 8, (**B**) 9, and (**C**) 10 as determined by gel elution assay. Insets show gel images of the pools eluted by various concentrations of THC.
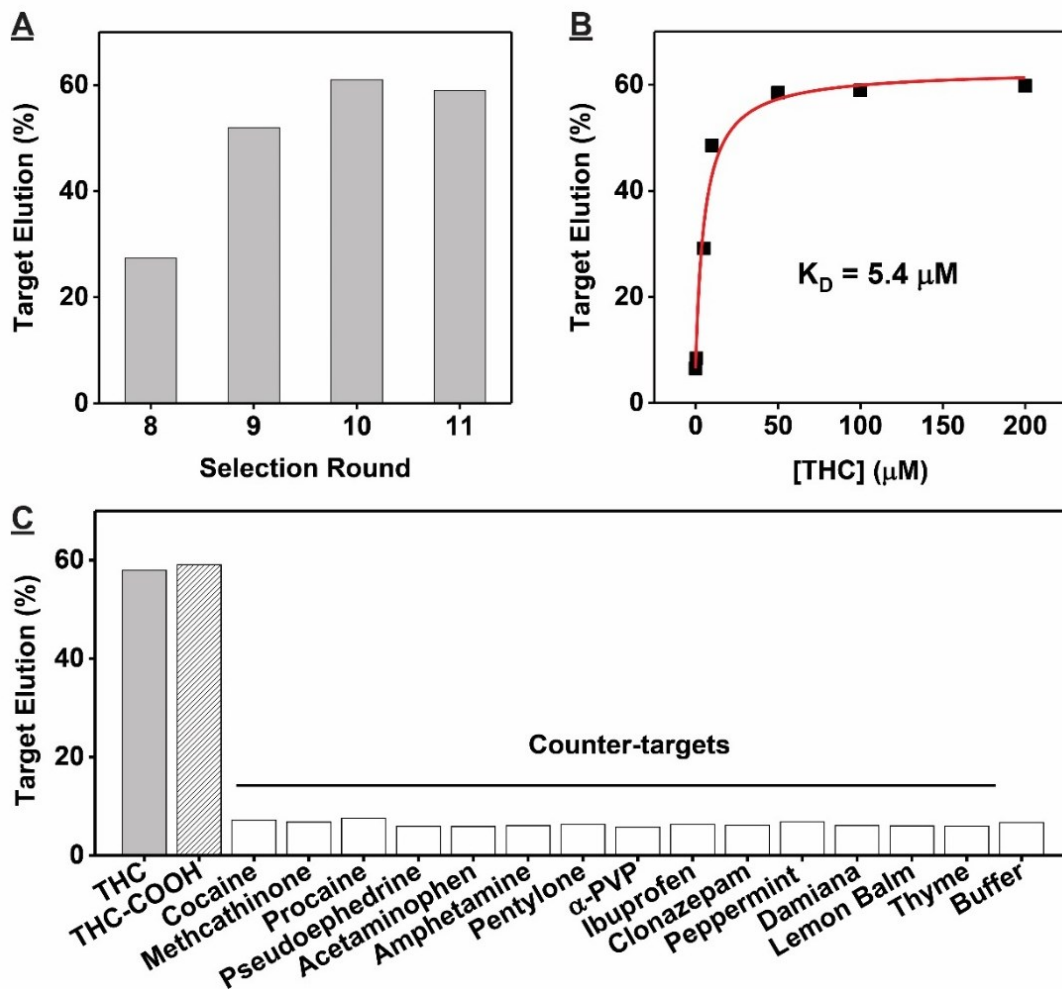
Figure S2. Characterization of SELEX THC pools using a gel elution assay. (**A**) Fraction of the enriched pools from rounds 8–11 eluted by 100 µM THC. (**B**) Fraction of the final round 11 pool eluted by 0, 5, 10, 25, 50, 100, or 200 µM THC was used to determine target-binding affinity. (**C**) Fraction of the round 11 pool eluted by 50 µM THC or THC-COOH; 300 µM cocaine, methamphetamine, procaine, pseudoephedrine, acetaminophen, amphetamine, pentylone, or α-pyrrolidinopentiophenone (α-PVP); 200 µM ibuprofen or clonazepam; or 0.16 mg/mL extracts of peppermint, damiana, lemon balm, or thyme.
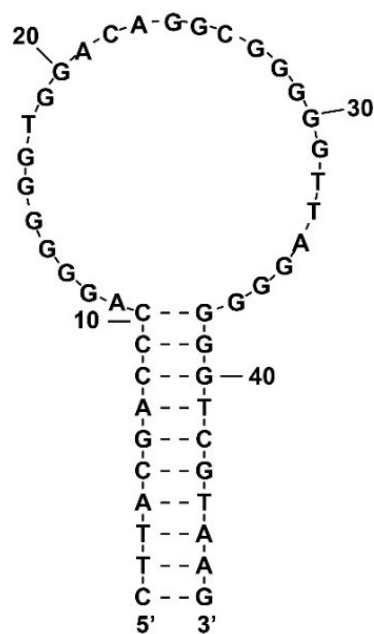
$\Delta G = -8.71$ kcal mol^{-1}

Figure S3. Secondary structure of THC1.2 and free energy as predicted by Mfold[5].
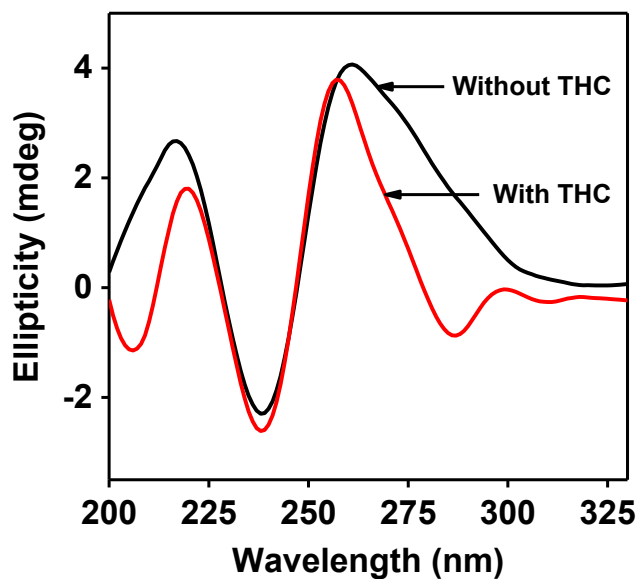
Figure S4. Circular dichroism spectra of THC1.2 in the absence (black) and presence (red) of 2 μM THC after subtracting spectra of reaction buffer without or with target, respectively.
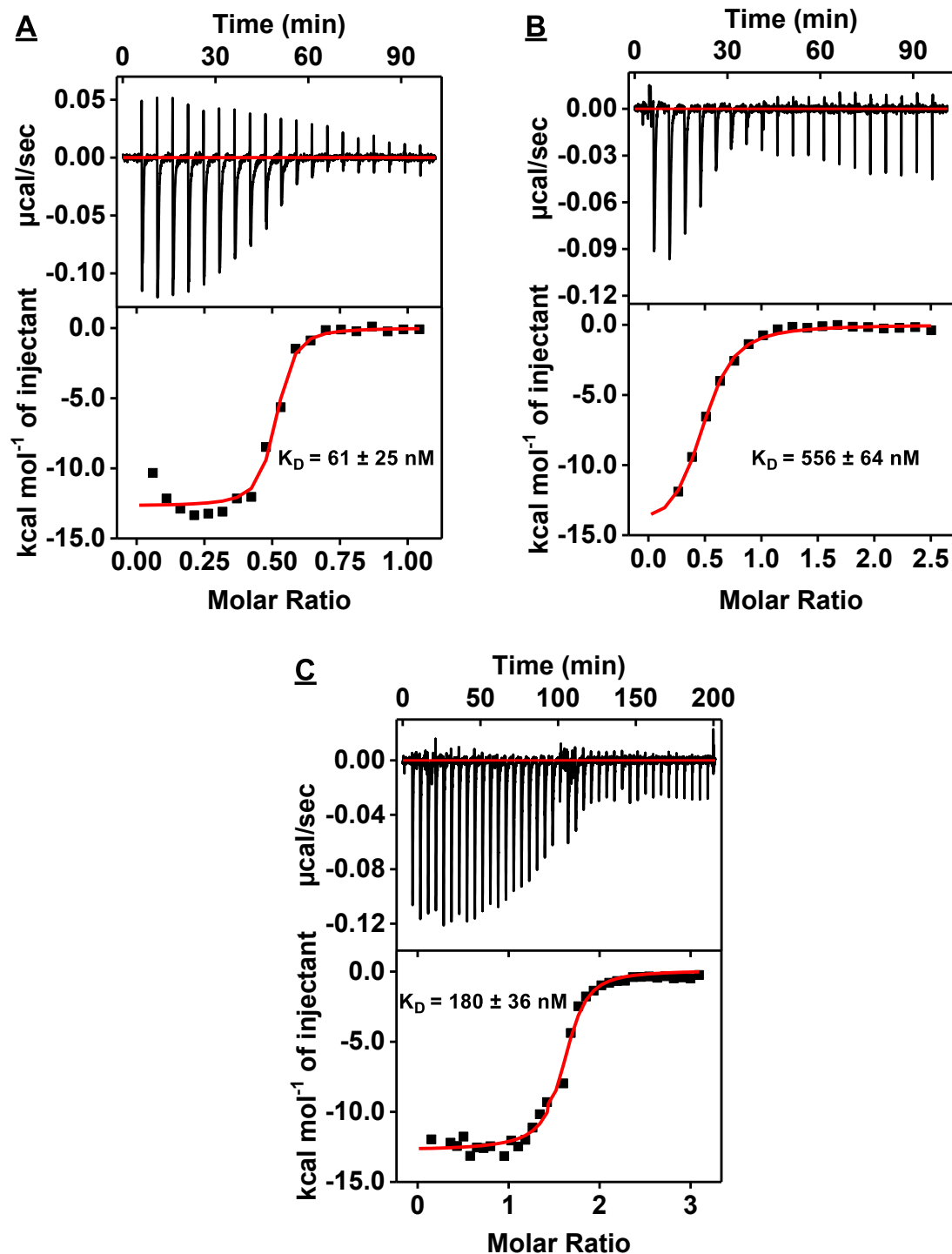
Figure S5. Characterization of the ligand-binding affinity of THC1.2 using ITC. Top panels present raw data showing the heat generated from each titration of (**A**) THC, (**B**) THC-OH or (**C**) THC-COOH to THC1.2, while bottom panels show the integrated heat of each titration after correcting for dilution heat of the titrant. Detailed ITC conditions are listed in **Table S5**. The data were fitted using a single-site model.
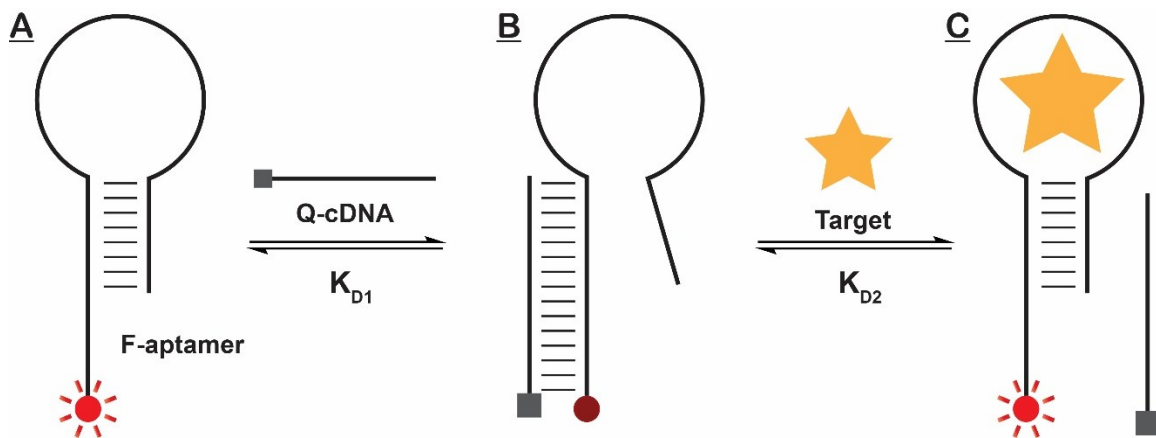
Figure S6. Schematic of the strand-displacement fluorescence assay used for determining the THC-binding affinity of THC1.2. K_{D1} is determined by titrating different concentrations of Q-cDNA into a solution of F-THC1.2. (**A**) When F-THC1.2 is free in solution, it emits strong fluorescence; (**B**) hybridization with Q-cDNA brings the quencher into close proximity to the fluorophore, greatly decreasing emission. K_{D2} is determined by titrating the various concentrations of THC into solutions of F-THC1.2-Q-cDNA complexes. (**C**) Target binding to the aptamer induces dissociation of Q-cDNA, resulting in recovery of fluorescence. Aptamer target affinity (K_D) is equal to K_{D1}/K_{D2}.
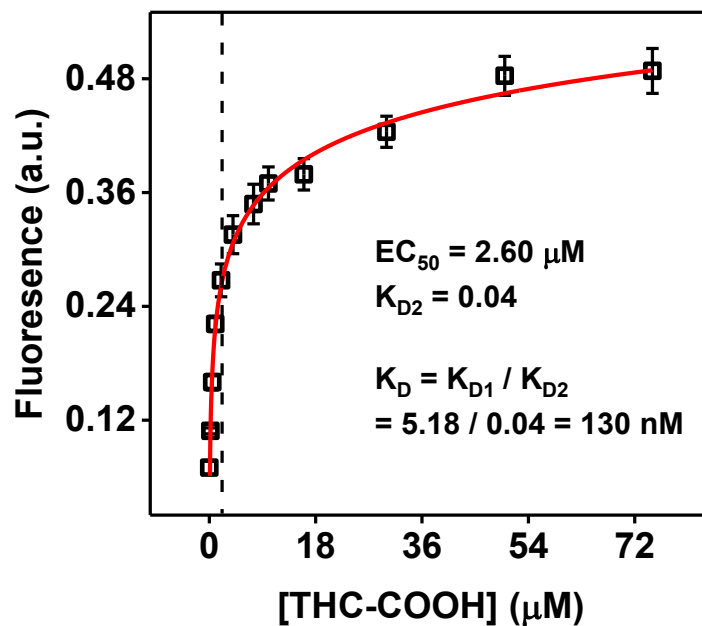
Figure S7. K_D of THC1.2 for THC-COOH based on a strand-displacement fluorescence assay. Error bars indicate the standard deviation of three measurements.
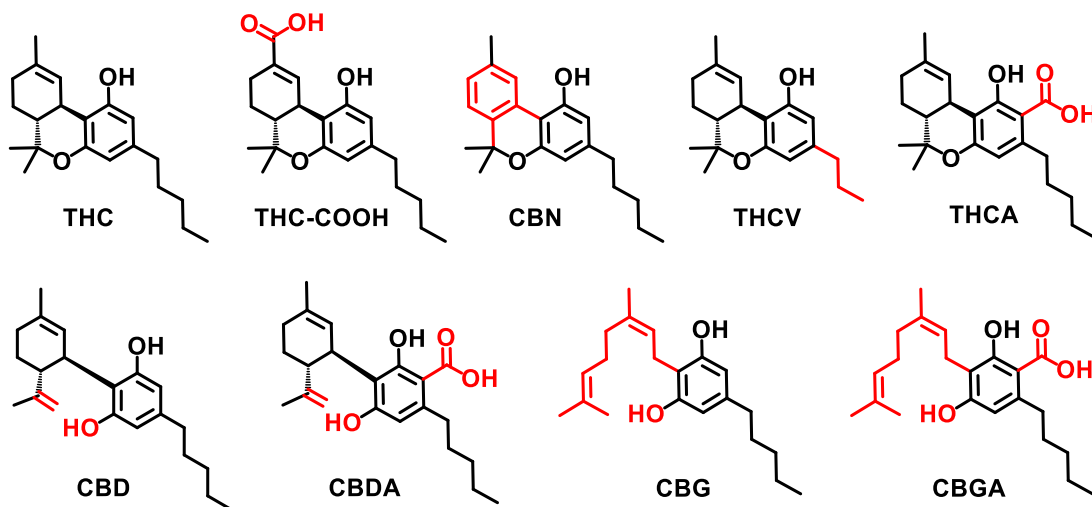
Figure S8. Chemical structures of different cannabinoids, with substituents relative to THC marked in red.
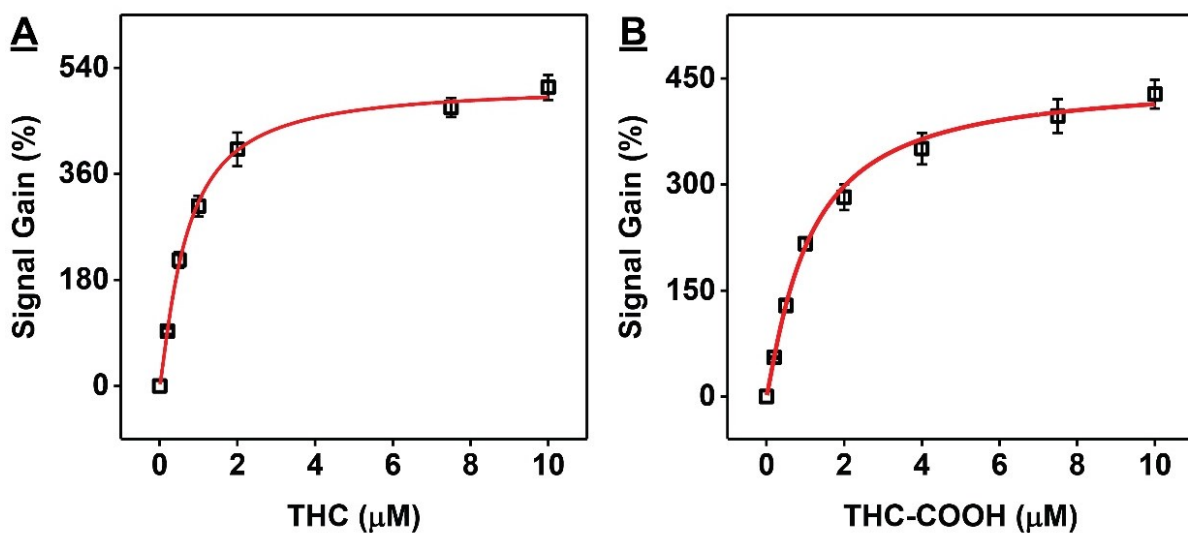
Figure S9. Calibration curve of THC1.2 for (**A**) THC and (**B**) THC-COOH based on the strand-displacement fluorescence assay. Error bars indicate the standard deviation of three measurements. The measurable detection limit is determined as the lowest non-zero concentration of target tested that produced a signal greater than three times its own standard deviation. The limit of detection is 200 nM for both analytes.
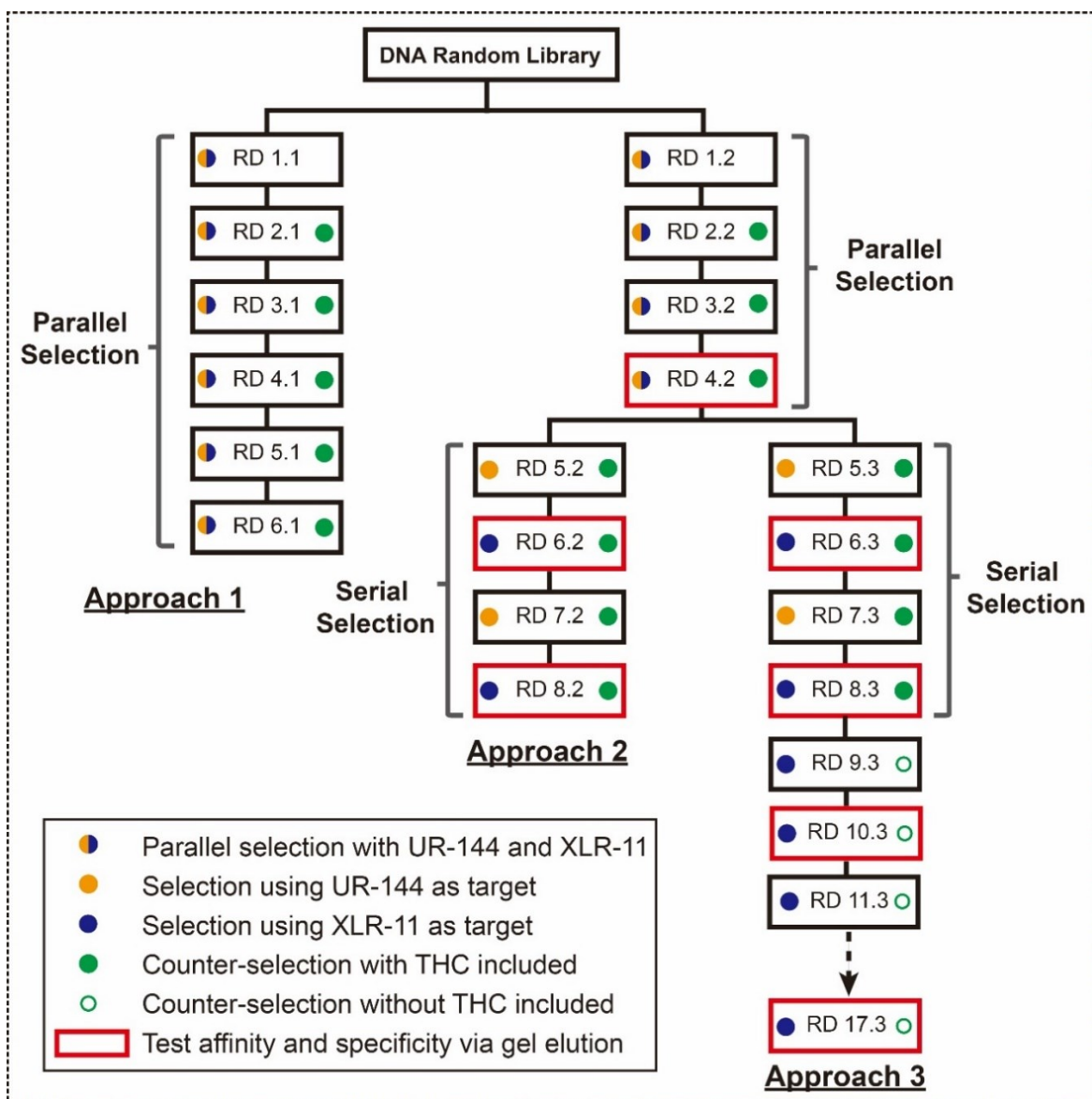
Figure S10. Three SELEX approaches for isolating an aptamer that binds UR-144 and XLR-11.
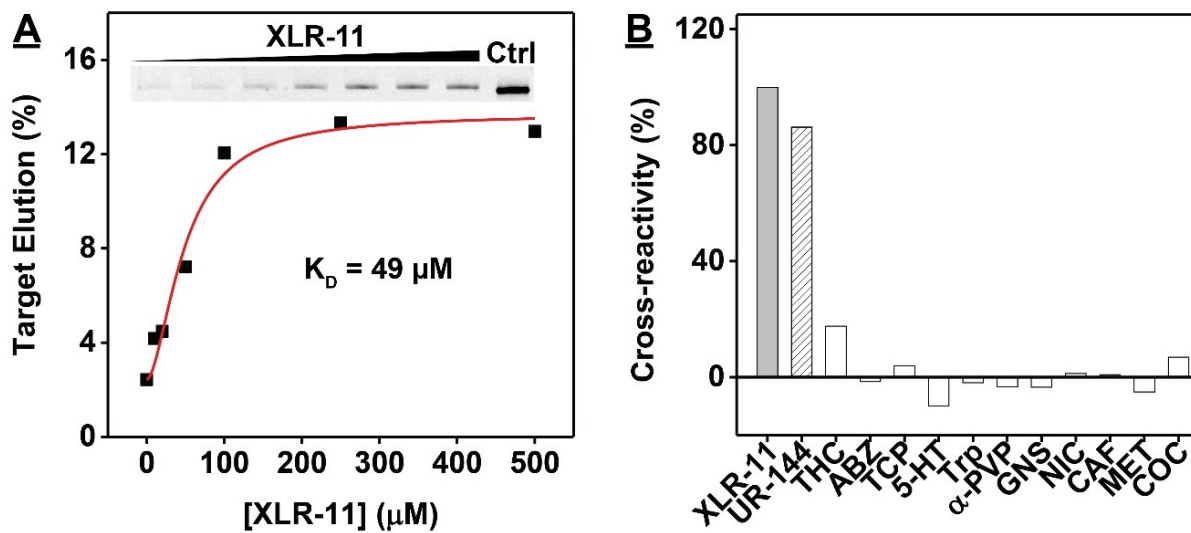
Figure S11. Characterization of the combined round 4.2 pool after four rounds of parallel selection for XLR-11. (**A**) XLR-11 affinity and (**B**) cross-reactivity were determined by gel elution assay.
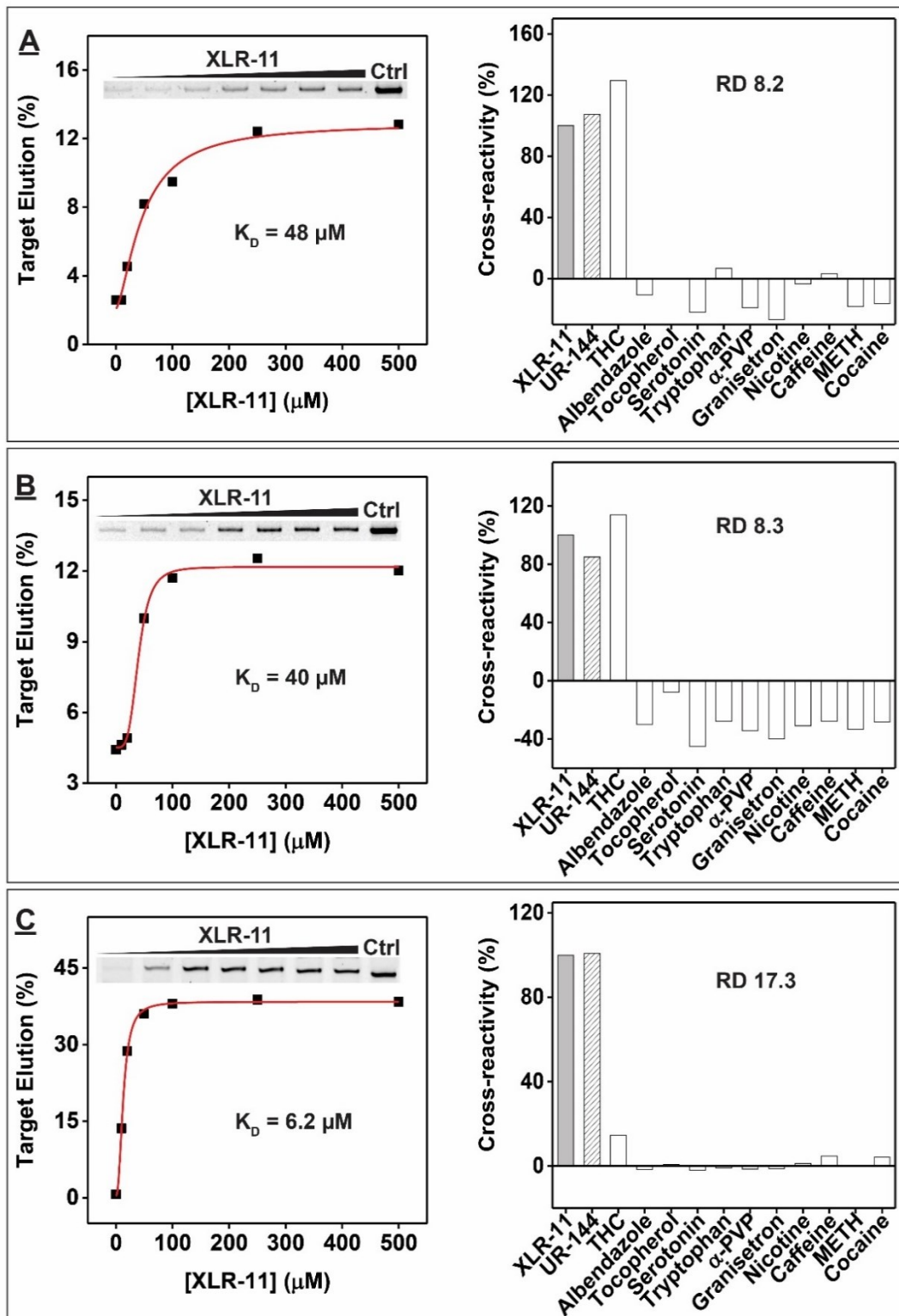
Figure S12. Characterization of pool affinity and specificity using a gel elution assay. XLR-11 binding affinity (left) and cross-reactivity (right) to UR-144 and counter-targets for the round (**A**) 8.2, (**B**) 8.3, and (**C**) 17.3 pools.
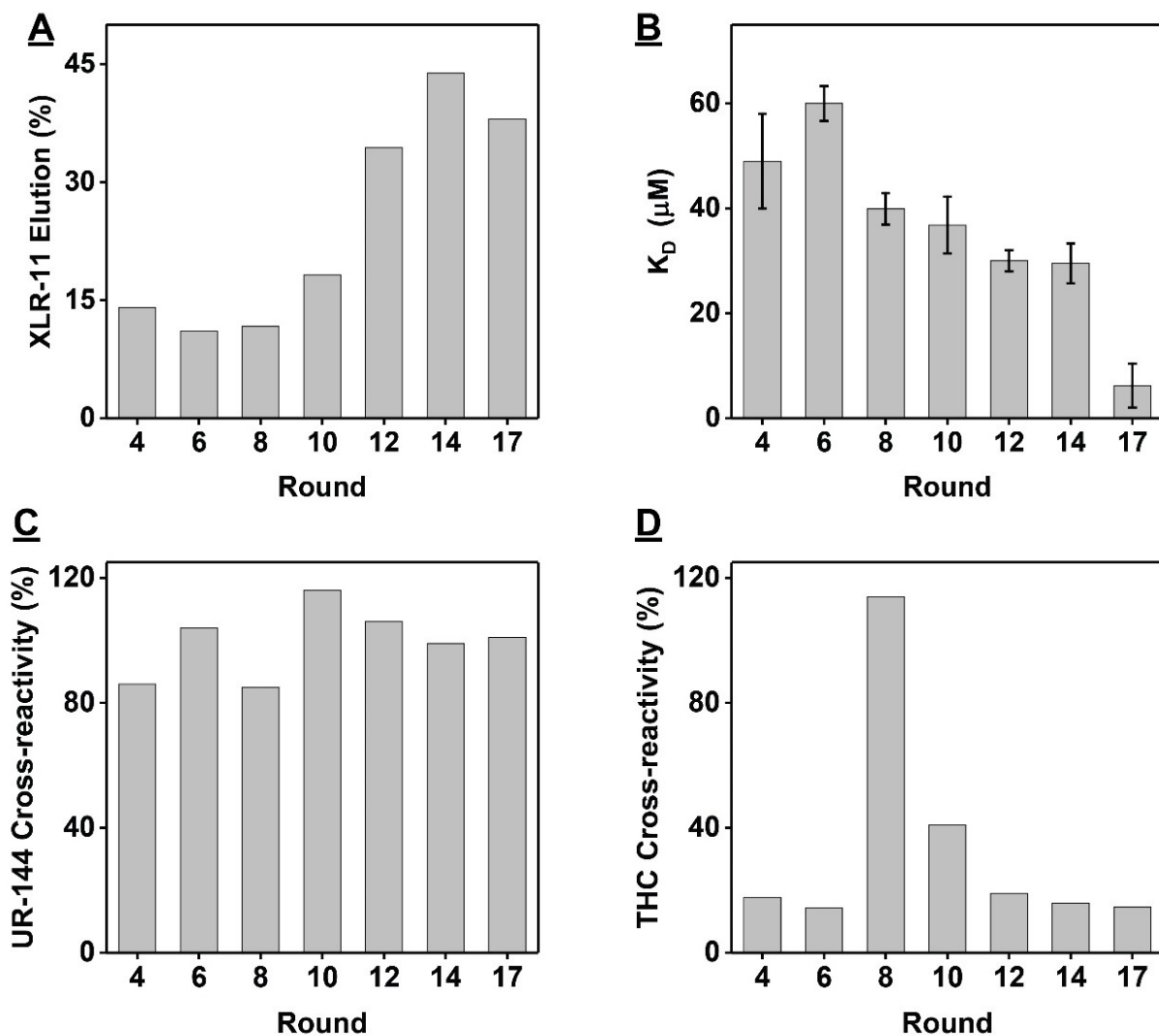
Figure S13. Selection progress for isolating an XLR-11/UR-144-binding aptamer. (**A**) XLR-11-induced pool elution, (**B**) XLR-11 affinity, and cross reactivity to (**C**) UR-144 and (**D**) THC for the round 4, 6, 8, 10, 12, 14, and 17 pools as determined by gel-elution assay. For K_D measurements, the error bar shows standard deviation from fitting with the Langmuir equation.
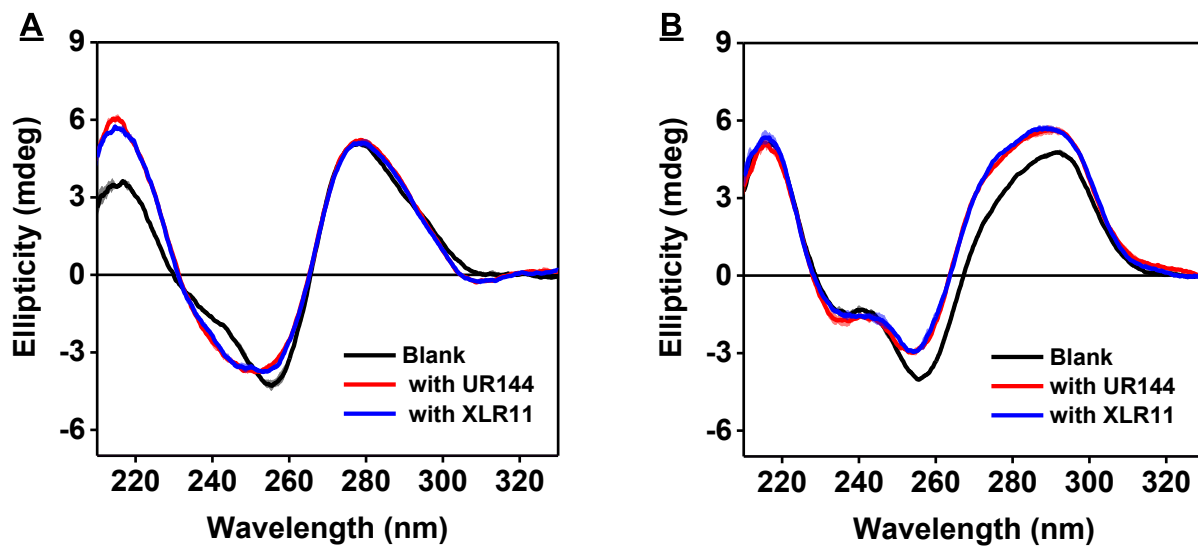
Figure S14. Circular dichroism spectra of XLR-11 binding aptamers (**A**) XA1 and (**B**) XA2 in the absence (black) and presence (red) of 10 µM XLR-11 or UR-144 after subtracting spectra of reaction buffer without or with target.
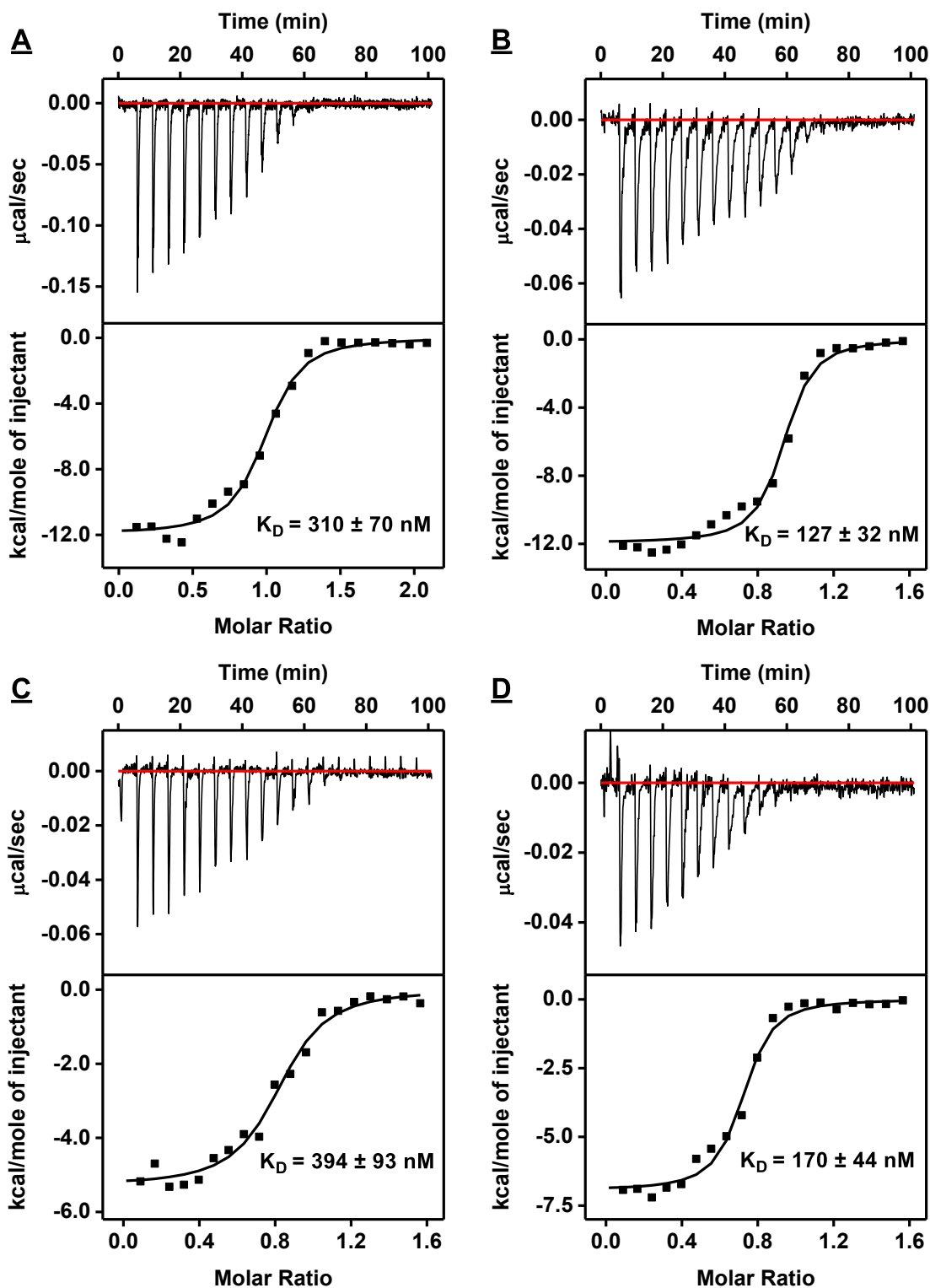
Figure S15. Characterization of target-binding affinity of XA1 and XA2 using ITC. Titration of (**A**) XLR-11 or (**B**) UR-144 into XA1, and (**C**) XLR-11 or (**D**) UR-144 into XA2. Detailed ITC conditions are listed in **Table S5**. ITC data were fitted using a single-site model.
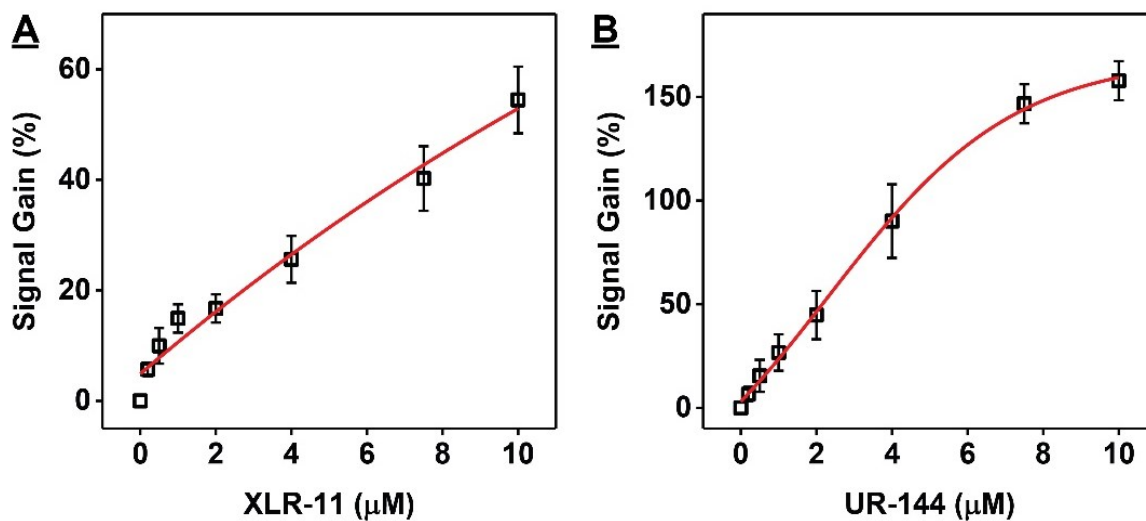
Figure S16. Calibration curve of XA1 for (**A**) XLR-11 and (**B**) UR-144 for the strand-displacement fluorescence assay. Concentrations greater than 10 μM were not tested due to the limited water solubility of these compounds. Error bars indicate the standard deviation of three measurements. The measurable detection limit is determined as the lowest non-zero concentration of target tested that produced a signal greater than three times its own standard deviation. The limit of detection for both XLR-11 and UR-144 is 0.2 μM.
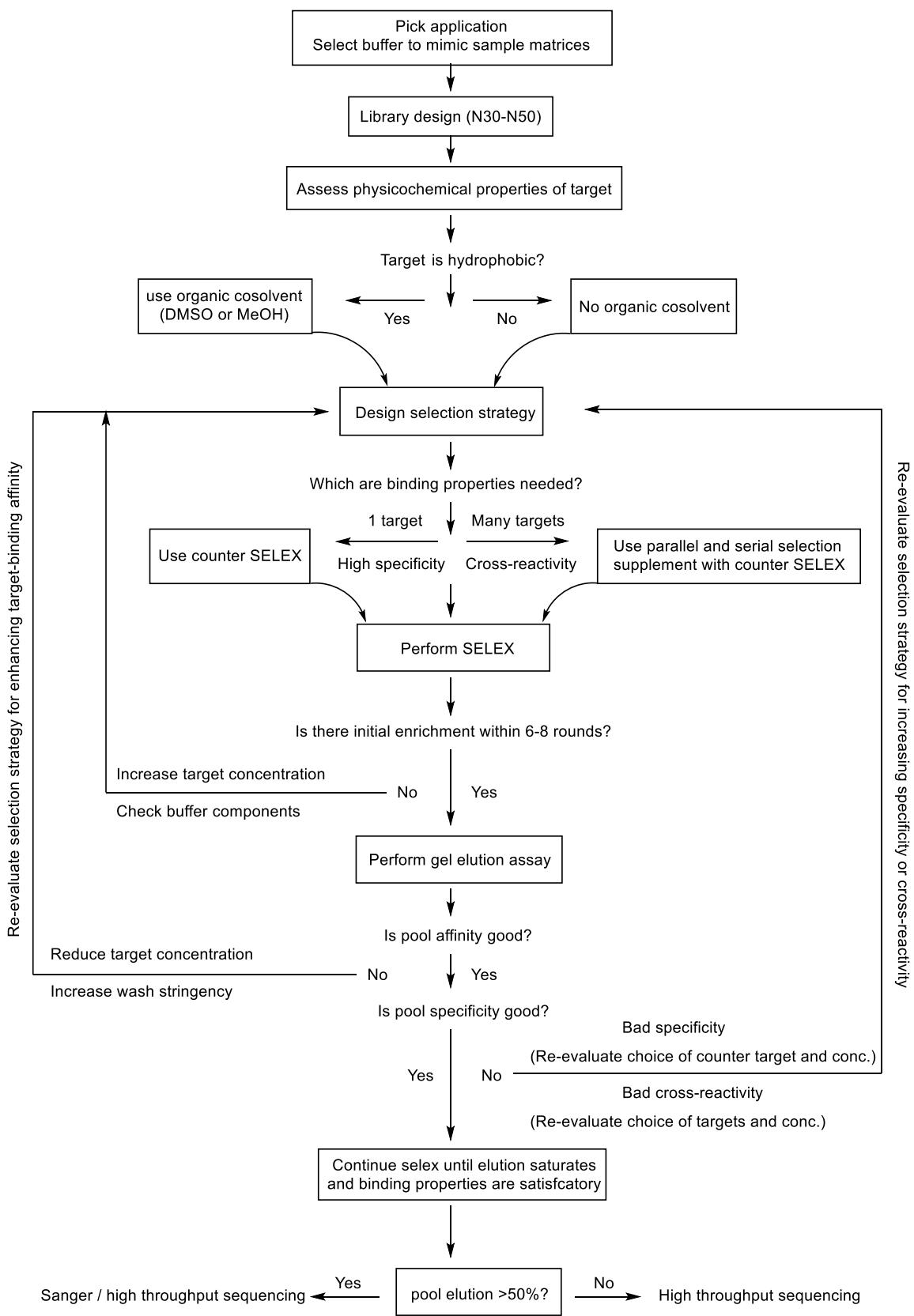
Figure S17. An algorithm to guide SELEX isolation of high-quality aptamers for small molecules.

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