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Rapid Nuclease-Assisted Selection of High-Affinity Small-Molecule Aptamers

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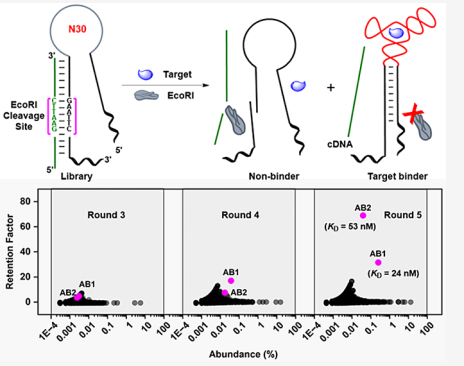
ABSTRACT: Aptamers are nucleic acid bioreceptors that have been widely utilized for a variety of biosensing applications, including *in vivo* detection methods that would not be possible with antibody-based systems. However, it remains challenging to generate high-quality aptamers for small molecule targets, particularly for use under physiological conditions. We present a highly effective aptamer selection technology for small-molecule targets that utilizes the nuclease *Eco*RI to remove nonspecific or weakly binding sequences in solution phase, rapidly enriching high-affinity target binders within just a few rounds of selection. As proof-of-concept, we used our nuclease-assisted SELEX (NA-SELEX) method to isolate aptamers for a synthetic cannabinoid, AB-FUBINACA. Within five rounds, we identified two highly specific aptamers that exhibit nanomolar affinity at physiological temperature. We also demonstrate the robustness and reproducibility of NA-SELEX by performing the same selection experiment with fresh reagents and libraries, obtaining the same two aptamers as well as two other high-quality aptamer candidates. Finally, we compare NA-SELEX against a conventional library-immobilized SELEX screen for AB-FUBINACA using the same screening conditions, identifying aptamers with 25−100-fold weaker affinity after 11 rounds of selection. NA-SELEX therefore could be an effective selection method for the isolation of high-quality aptamers for small-molecule targets.



INTRODUCTION

Aptamers are short oligonucleotide-based affinity reagents that are isolated from randomized libraries through *in vitro* selection methods such as systematic evolution of ligands by exponential enrichment (SELEX).[1,2] Aptamers offer numerous advantages as biorecognition elements for sensing applications, including high stability, low cost, ease of handling, minimal batch variation, and amenability to chemical labeling and sequence engineering.[3,4] In the context of small-molecule analyte detection, aptamer-based sensors have been developed for a variety of applications including medical diagnostics, environmental monitoring, forensics, and food safety.[5] Recent research has even demonstrated that aptamers can deliver capabilities that were not previously achievable with conventional antibody-based sensing modalities, including the continuous monitoring of small molecules *in cellulo*[6] and *in vivo* in real time.[7]

Despite their promise, the implementation of aptamers for sensing has been hindered by the difficulty of generating aptamers with sufficiently high target affinity and specificity. There are several reasons why this task remains challenging. First, small-molecule targets have fewer functional groups that can interact with aptamers relative to protein targets. Therefore, aptamers that bind small molecules tend to have dissociation constants in the micromolar range on average.[8] Second, most existing methods for isolating small-molecule binding aptamers are inefficient and prone to failure. The first small-molecule-binding aptamers were isolated by immobilizing the target onto a solid surface to facilitate the separation of binding aptamers from nonbinding sequences.[1] Although these approaches have proven successful to some extent,[9,10] they typically yield aptamers with micromolar binding affinity because surface-immobilization excludes interactions with a sizable portion of the target. In addition, the generality of these methods is limited, as not all small molecules are amenable to surface attachment since they can lack conjugatable moieties. As an alternative, some groups have instead immobilized aptamer libraries onto streptavidin agarose resin using biotinylated oligonucleotides that are complementary to a specific region of the library.[11,12] With this approach, the small-molecule target can freely interact with the immobilized aptamers, enabling less impeded aptamer-target interaction. Indeed, several high-affinity aptamers for small molecules have been discovered using this method.[13,14] Nevertheless, the major disadvantage of library-immobilized SELEX is that the efficiency of separating aptamers from nonbinding sequences is

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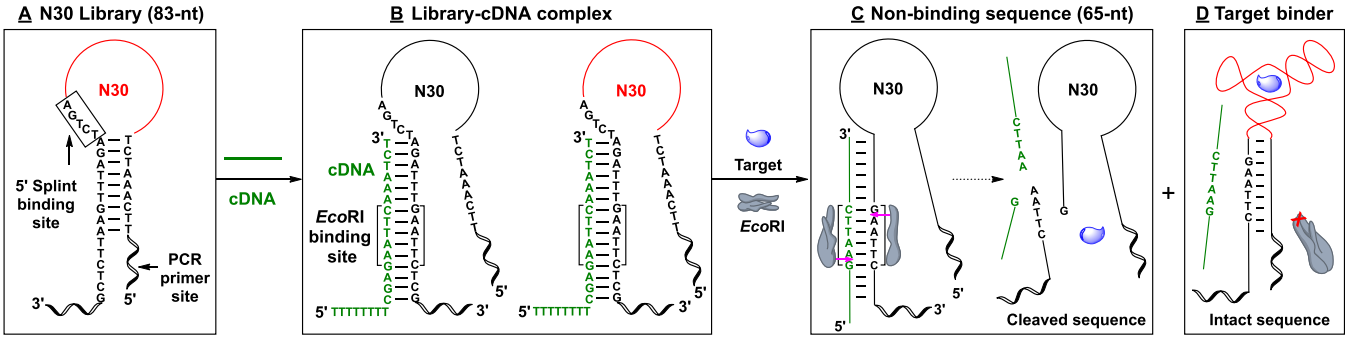

Figure 1. Structure of the library and cDNA employed in NA-SELEX and the mechanism of *Eco*RI-based aptamer selection. (A) Nucleotide sequence of the 83-nt N30 library (the splint binding site is boxed) and (B) the library hybridized to cDNA15 to form a library-cDNA complex. *Eco*RI binding site and cleavage sites are highlighted. (C) Library sequences that do not bind the target are cleaved by *Eco*RI while (D) target-binders dissociate from the cDNA, resulting in the dismantling of the *Eco*RI cleavage site and escaping from enzymatic digestion.

remarkably low. It is commonplace for such selections to take as many as 20 rounds, with an average completion time of 14 rounds (Table S1 and Figure S1). This can be attributed to the consistent nonspecific elution of library sequences due to spontaneous dissociation from the complementary sequence. As such, desired aptamers are largely drowned out by the background elution of nonbinding sequences. An additional drawback of this spontaneous dissociation is the stochastic loss of high-quality aptamers, which can become extinct especially in early rounds when their copy numbers are low. Third, both target- and library-immobilized selection methods can only be practically carried out under ambient conditions. Since these aptamers typically exhibit greatly reduced affinity at physiological temperatures (37 °C),[15] they are ineffective for *in vivo* biomedical applications or experiments in cultured cells.

Here, we present a new solution-phase method termed nuclease-assisted SELEX (NA-SELEX) that uses enzymes to efficiently isolate DNA aptamers with high affinity and specificity for small-molecule targets at 37 °C with only a few rounds of selection. This method uses a specially designed stem-loop structured library that hybridizes with a complementary DNA strand to form a recognition site for the DNA nuclease *Eco*RI.[16] For nonbinding sequences, these library-cDNA complexes are efficiently cleaved by *Eco*RI. In contrast, sequences that bind the target concomitantly dissociate from the complementary DNA, thereby dismantling the *Eco*RI site and permitting such sequences to avoid digestion. These intact sequences can be recovered, and PCR amplified for another round of selection. NA-SELEX has several advantages relative to conventional SELEX approaches. First, NA-SELEX allows for completely unimpeded interaction of the library and target because it is performed entirely in solution, thereby promoting the enrichment of high affinity binders. Second, NA-SELEX has greater separation efficiency due to its continuous removal of nonbinding sequences, which thereby leads to fewer rounds required to enrich aptamers. In contrast to library-immobilized selection, in which the eluent from each round is contaminated by spontaneously dissociated nonspecific sequences, such nonbinding sequences can be in theory completely removed during NA-SELEX. This is because enzymatic digestion continuously drives equilibrium toward the formation of library-cDNA complexes, which will eventually consume all nonbinding sequences. Third, NA-SELEX provides additional selection stringency (high temperature) that is hard to achieve with conventional selection strategies. Since target-binding and sequence separation during NA-SELEX is performed at 37 °C rather than room temperature, only the highest affinity binders can survive the selection process. Such aptamers, which can function well at physiological temperatures, are ideal receptors for *in vivo* applications and can even serve as ultrahigh affinity receptors for point-of-care applications performed at ambient conditions.

As proof-of-concept, we employed NA-SELEX to isolate DNA aptamers for AB-FUBINACA, an illicit synthetic cannabinoid of the indazole-3-carboxamide family.[17] The ability to detect AB-FUBINACA rapidly in biofluids is important because this drug can cause severe adverse effects. We were able to identify high affinity aptamers after performing only five rounds of NA-SELEX—to the best of our knowledge, this is among the most rapid small-molecule aptamer selection experiment described to date. Notably, these aptamers had target-binding affinities in the nanomolar range, with an average K_D of 32 nM at room temperature and 523 nM at 37 °C per isothermal titration calorimetry (ITC). More impressively, the aptamers demonstrated excellent specificity, with minimal or no response to interferents such as illicit drugs, adulterants, cutting agents, and endogenous substances. To confirm the reproducibility of our technique, we performed the same NA-SELEX experiment a year later with resynthesized oligonucleotides and new reagents, and were able to identify the same high affinity aptamer after five rounds as well as a few new high-affinity aptamers. As a comparison, we performed conventional library-immobilized SELEX using the same library, target, and selection conditions at room temperature. After 11 rounds, we were only able to identify aptamers with 25−110-fold poorer affinity, and did not obtain any of the aptamers discovered via NA-SELEX. Finally, to demonstrate the utility of the isolated aptamers, we developed a strand-displacement fluorescence sensor that detected AB-FUBINACA in human serum at nanomolar concentrations.

RESULTS

NA-SELEX Approach. NA-SELEX utilizes an 83-nucleotide (nt) stem-loop structured DNA library (Table S2) wherein each oligonucleotide member contains a constant nine-base pair (bp) stem followed by a 30-nt random region serving as the putative target binding domain, with flanking PCR primer sites at both termini (Figure 1A). Downstream of the random domain is a 5-nt constant sequence that serves as an initiation site for Klenow fragment,[18] which is used to regenerate library sequences after an initial negative selection step (described below). A complementary DNA (cDNA15) is

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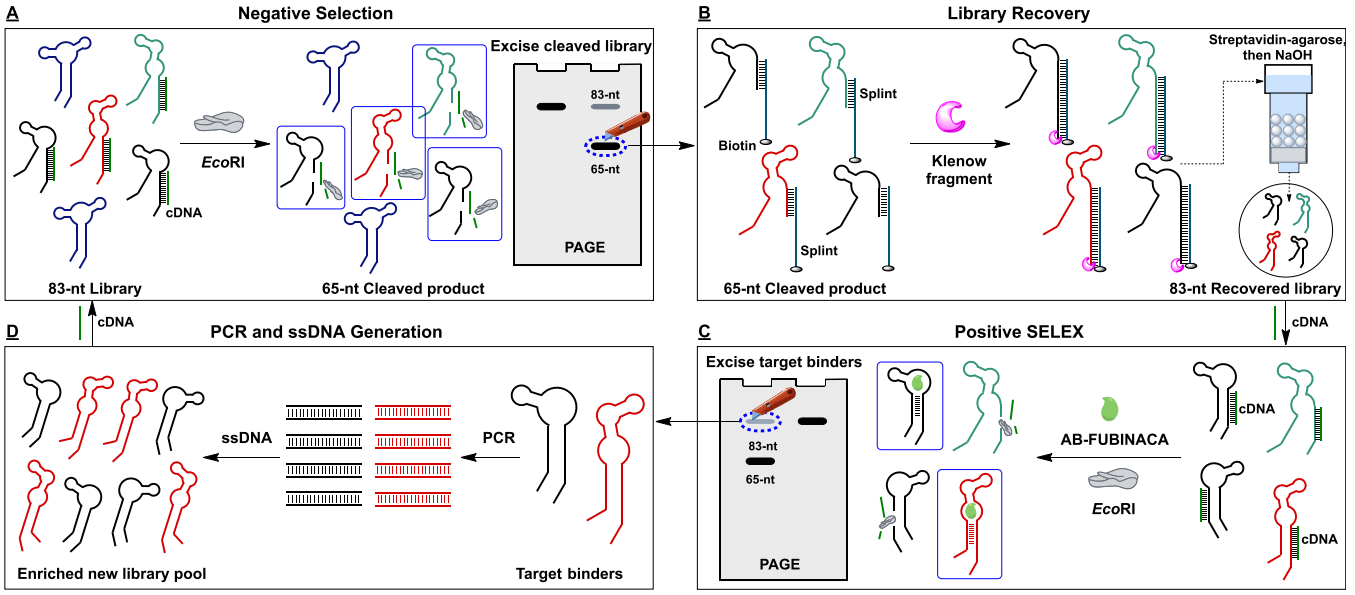

Figure 2. Scheme of NA-SELEX. (A) The library is first subjected to negative selection by digesting library-cDNA complexes in the absence of target with *Eco*RI. Digestible sequences are recovered using PAGE purification. (B) The digested library is restored to its original length using a biotinylated splint hybridized to a constant region of the aptamer strands and the Klenow fragment. These duplexes are then immobilized onto streptavidin-coated agarose resin, and aptamers are released from the resin using sodium hydroxide. This solution is pH-neutralized and desalted. (C) The recovered library is then hybridized with the cDNA and subjected to positive selection with the target. Nonbinders are efficiently digested by *Eco*RI, whereas target-binders remain intact and are purified by PAGE. (D) Intact aptamers are PCR amplified and then converted to single-stranded DNA (ssDNA) for the next round of selection.

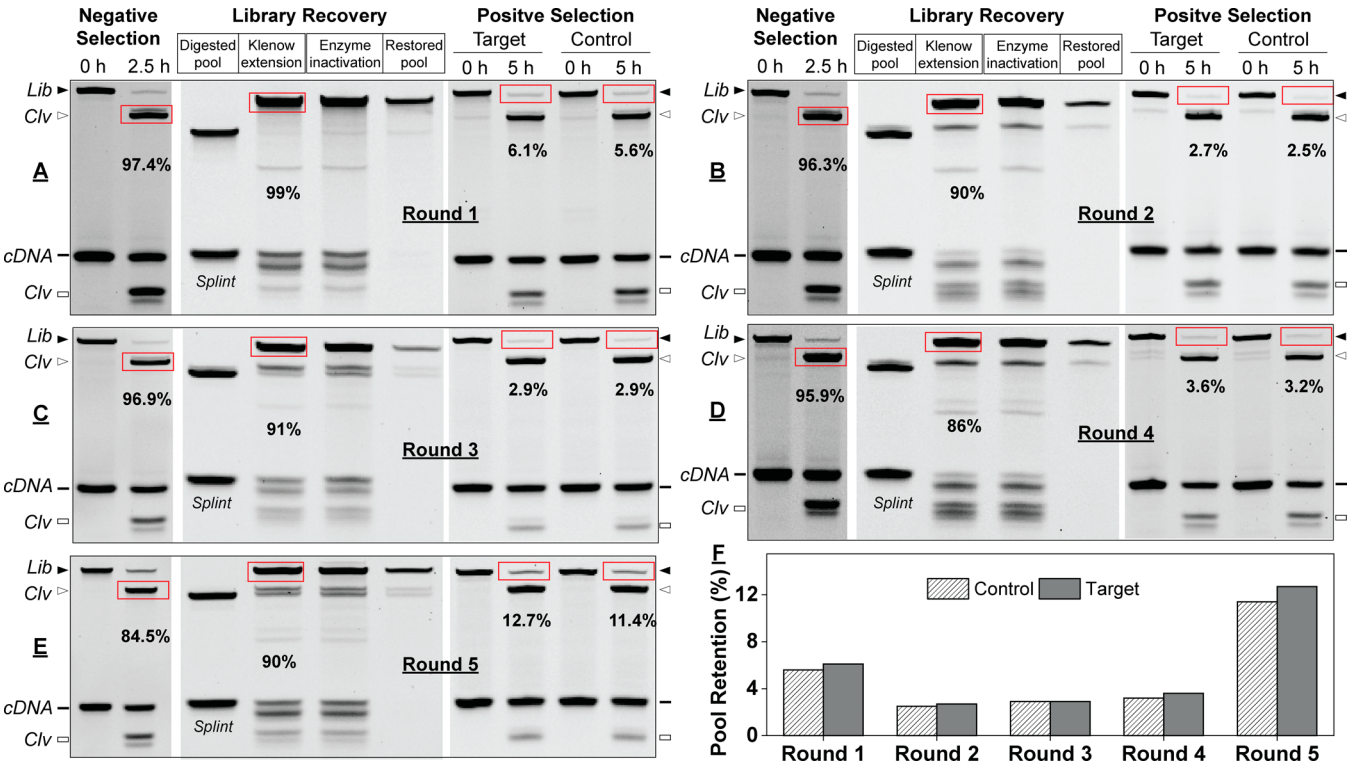

Figure 3. Results of NA-SELEX for the isolation of aptamers for the small molecule target, AB-FUBINACA. PAGE analysis of negative selection, library elongation by Klenow fragment, and positive selection in the presence and absence of target (control) for (A) Round 1, (B) Round 2, (C) Round 3, (D) Round 4, (E) Round 5. Black and open triangles represent intact and cleaved library, respectively. Black and open rectangles represent intact and cleaved cDNA, respectively. (F) Pool retention for each round of NA-SELEX after digestion of library with *Eco*RI in the absence (control) or presence of target.

hybridized with 15 base pairs near the 3′ terminus of the library strand, forming a recognition site for the restriction endonuclease *Eco*RI (Figure 1B). Nontarget-bound oligonucleotide strands retain the cleavage site and are digested after

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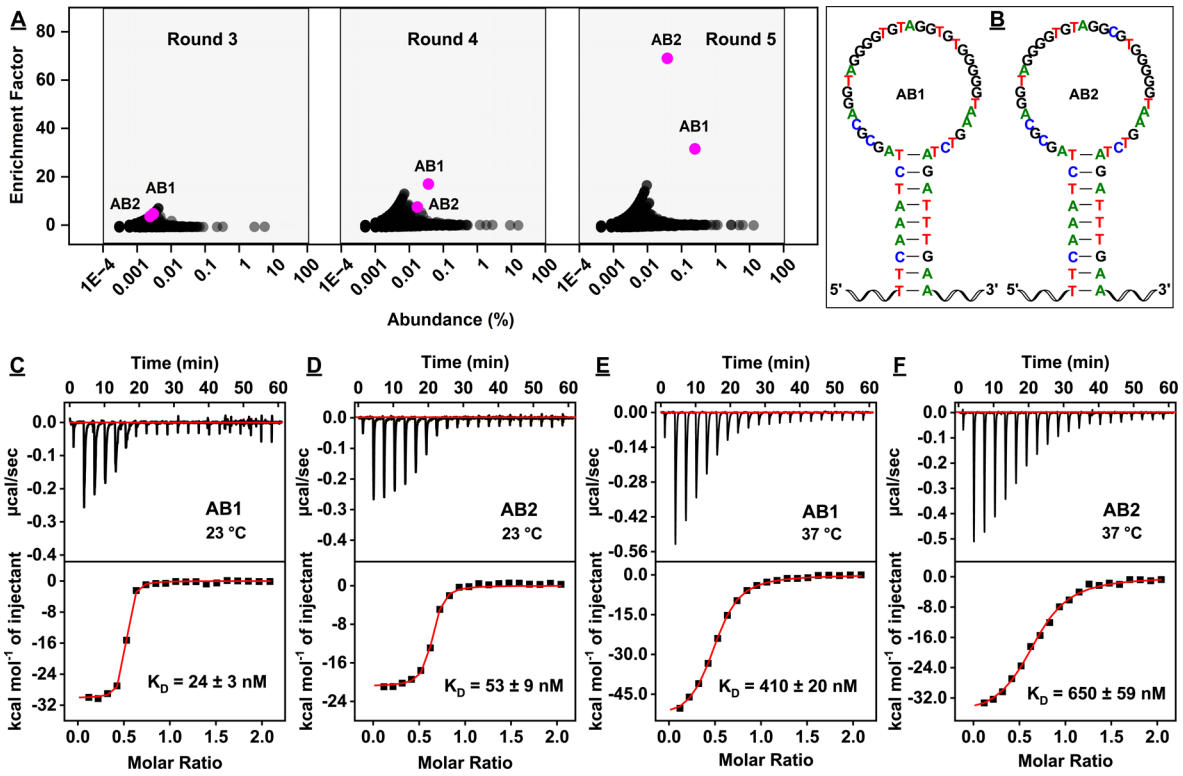

Figure 4. HTS analysis of NA-SELEX pools and aptamer characterization. (A) Retention factor plotted against abundance for each sequence in Rounds 3, 4, and 5 of NA-SELEX. Each data point represents a unique sequence, and aptamers AB1 and AB2 are indicated as purple dots. (B) Secondary structure of AB1and AB2 as predicted by NUPACK. AB2 is a one-nucleotide mutant of AB1. K_D values of AB1 and AB2 for AB-FUBINACA were determined using ITC at (C, D) 23 °C and (E, F) 37 °C.

incubation with this enzyme, forming a shortened 65-nt product (Figure 1C). In contrast, aptamers that bind the target dissociate from cDNA15, disrupting the *Eco*RI cleavage site and thereby keeping these aptamers intact (Figure 1D). These target-bound aptamers are separated from cleaved nonbinding sequences via polyacrylamide gel purification, PCR amplified and converted into a new single-stranded pool for another round of selection.

We performed NA-SELEX using the designer drug AB-FUBINACA, a hydrophobic synthetic cannabinoid, to demonstrate this approach. Each round of NA-SELEX entails an initial negative selection step, followed by library regeneration with Klenow fragment, and finally positive selection with the target. Negative selection is performed to eliminate nontarget-binding sequences that are not digestible by *Eco*RI (Figure 2A). Specifically, the library is hybridized with a 5-fold excess of cDNA15 via heat treatment and gradual cooling. Then, the library-cDNA complexes are digested with *Eco*RI, after which digestion is quenched by denaturing the enzyme with heat. Polyacrylamide gel electrophoresis (PAGE) is used to separate the cleaved 65-nt library strands from the intact 83-nt library (Figure 2A). To restore the cleaved library to its original length prior to positive selection, the cleaved library strands are hybridized with a biotinylated 30-nt splint at their 3′ end. The splint partially hybridizes with the cleaved library strand and allows the enzyme Klenow fragment to elongate the cleaved strand back to its original full length. The resulting double-stranded constructs are captured on streptavidin-immobilized agarose beads, and the aptamer strand is released by treating the beads with sodium hydroxide (Figure 2B). This resulting pool is then subjected to positive selection by annealing with cDNA15. The pool is split into two equal portions, one of which is incubated with AB-FUBINACA and the other with buffer as a control, and both samples are digested with *Eco*RI. After deactivating the enzyme, PAGE is performed to separate intact from cleaved library strands (Figure 2C). The purified intact aptamers are PCR amplified, and the amplicons are converted to a single-stranded library for a subsequent round of NA-SELEX (Figure 2D).

Aptamer Isolation via NA-SELEX. For the first round of NA-SELEX (see Table S3 for conditions), we initiated negative selection by hybridizing 2 nmol of library ($\sim 10^{15}$ unique sequences) with a 5-fold excess of cDNA15 and then digesting with *Eco*RI. After 2.5 h, 97% of the library was digested by *Eco*RI (Figure 3A, negative selection). Extension of the cleaved 65-nt library back to the original 83-nt length with the Klenow fragment proved effective, with 99% of the library restored to full length (Figure 3A, library recovery). The recovered 83-nt library was then hybridized with cDNA15, challenged with 100 μM AB-FUBINACA or buffer as a control, and digested with *Eco*RI, where we observed only 6.1% of the library was retained with target, relative to 5.6% when digestion was performed without target (Figure 3A, positive selection). We utilized PAGE to selectively purify the intact library and then performed PCR to amplify the resulting sequences. In the next three rounds, 96−97% of the library was cleaved during negative selection, 86−91% was recovered by Klenow fragment extension, and 2.7−3.6% of the library was retained in the presence of target for positive selection versus 2.5−3.2% in the absence of target (Figure 3B−D). In Round 5, we observed a considerable increase in pool retention during positive selection, with 12.7% of the library retained in the presence

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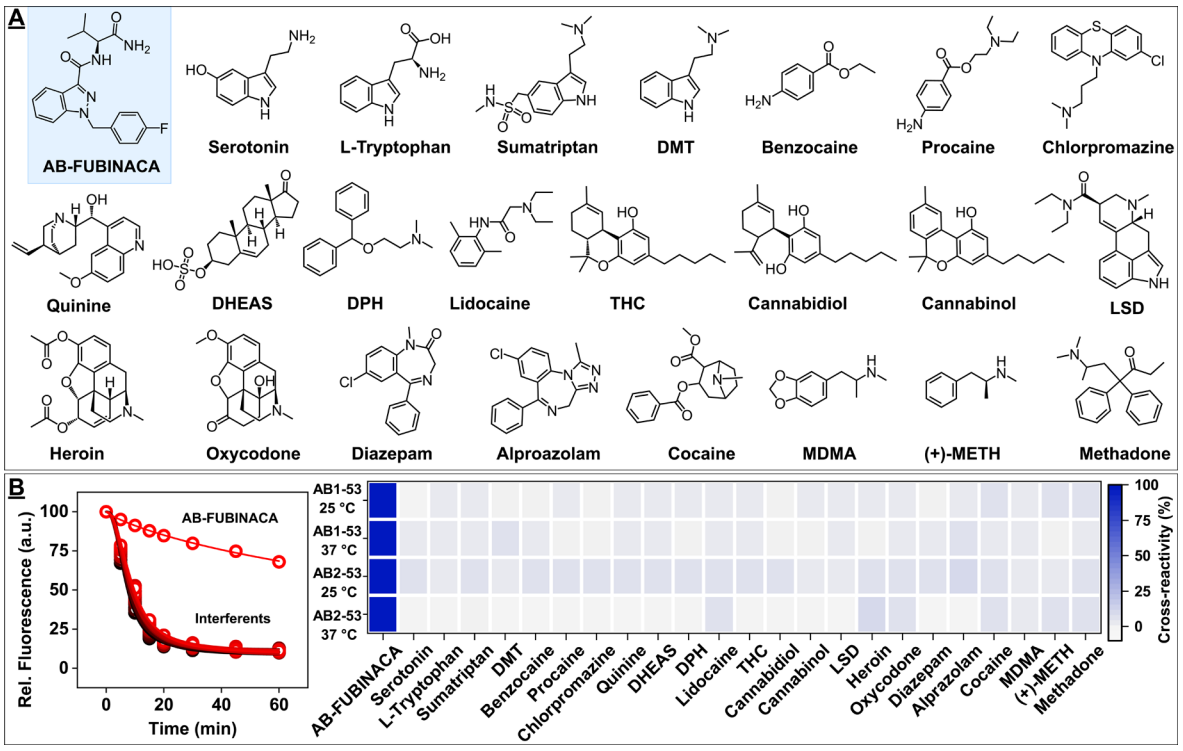

Figure 5. Specificity of AB1-53 and AB2-53 as determined using an exonuclease digestion fluorescence assay. (A) Chemical structures of AB-FUBINACA and the interferents used in the specificity test. (B) Lefthand plot shows the time-course of exonuclease digestion of AB1-53 at 25 °C over time in the absence of ligand and in the presence of AB-FUBINACA or an interferent. No interferent produced resistance levels that were meaningfully higher than the blank. Righthand heatmap summarizes the specificity data derived from resistance values. Cross-reactivity was calculated relative to AB-FUBINACA. The white-to-blue color gradient indicates increasing cross-reactivity.

of target versus 11.4% in the absence of target (Figure 3E). At this point, we concluded the NA-SELEX trial (Figure 3F) and subjected the pools to high-throughput sequencing (HTS).

HTS Analysis of NA-SELEX. To study the evolution of sequences during NA-SELEX, we subjected the Round 3, 4, and 5 pools to HTS. For each round, we sequenced pools after digestion with *Eco*RI in the absence (control pool) or presence of AB-FUBINACA, obtaining 182,000−332,000 reads per pool (Table S4). For AB-FUBINACA pools, the proportion of unique sequenes decreased from 36.7% in Round 3 to 18% in Round 5, indicating enrichment of specific sequences (Figure S2). Control pools also displayed a decrease in diversity over Round 3 to Round 5. The 10 most abundant sequences made up 34% of the Round 5 AB-FUBINACA pool (Table S5). However, these same sequences had similar relative populations in the control pool as well, suggesting that they may not have target-binding ability. To determine whether this was true, we performed ITC for these ten sequences, and confirmed that none of them had meaningful target affinity (Table S5). We therefore developed the "retention factor" metric to identify putative aptamer candidates, which is calculated using the following equation: $(P - P_0)/P_0$, where P is the population of a particular sequence in the target-selection pool and P_0 is the population of that sequence in the control pool. In theory, aptamers that bind the target should have high P and low P_0, and hence a large retention factor, while weak or nonbinding sequences should have similar P and P_0 values and thus a retention factor near zero. We determined that the 10 most abundant sequences had retention factors of −0.08 to 0.15 (Table S5), indicating low likelihood of functionality, which is corroborated by the ITC data. This data indicates that *Eco*RI was most likely unable to eliminate certain nonbinding sequences from the library, despite our presumption. However, we noted two sequences with exceptionally high retention factor values of 32 and 69 in the Round 5 AB-FUBINACA pool, which we respectively termed AB1 and AB2. HTS analysis indicated that the abundance and retention factor for these sequences continuously increased over Round 3, 4, and 5. In particular, AB1 and AB2 grew in abundance by 10-fold between Round 3 and Round 4, and by 8- and 2-fold between Round 4 and Round 5, respectively. For AB1, the retention factor increased from 4.7 to 17 and finally 32 over these three rounds, while the retention factor for AB2 increased from 3.6 to 7.5 to 69. When we plotted the retention factor of each sequence in each round versus abundance, AB1 could be clearly distinguished from the rest of the library by as early as Round 4, while AB2 stood out prominently in Round 5 (Figure 4A). We thus concluded that AB1 and AB2 could be specific binders to AB-FUBINACA.

Characterization of NA-SELEX Aptamers. We next assessed the binding properties of AB1 and AB2 (Figure 4B). First, we digested these sequences with *Eco*RI in the presence of cDNA15 and observed that both aptamers displayed significant resistance to digestion in the presence of AB-FUBINACA relative to the absence of target (Figure S3). This indicated that these two sequences bind AB-FUBINACA. We then performed ITC to measure the binding affinities of these sequences to this target in our selection buffer, and determined that AB1 and AB2 respectively had K_Ds of 24 ± 3 nM and 53 ± 9 nM at 23 °C (Figure 4C,D) and 410 nM ± 20 nM and 650 nM ± 59 nM at 37 °C (Figure 4E,F). These data confirmed that NA-SELEX can yield high affinity aptamers

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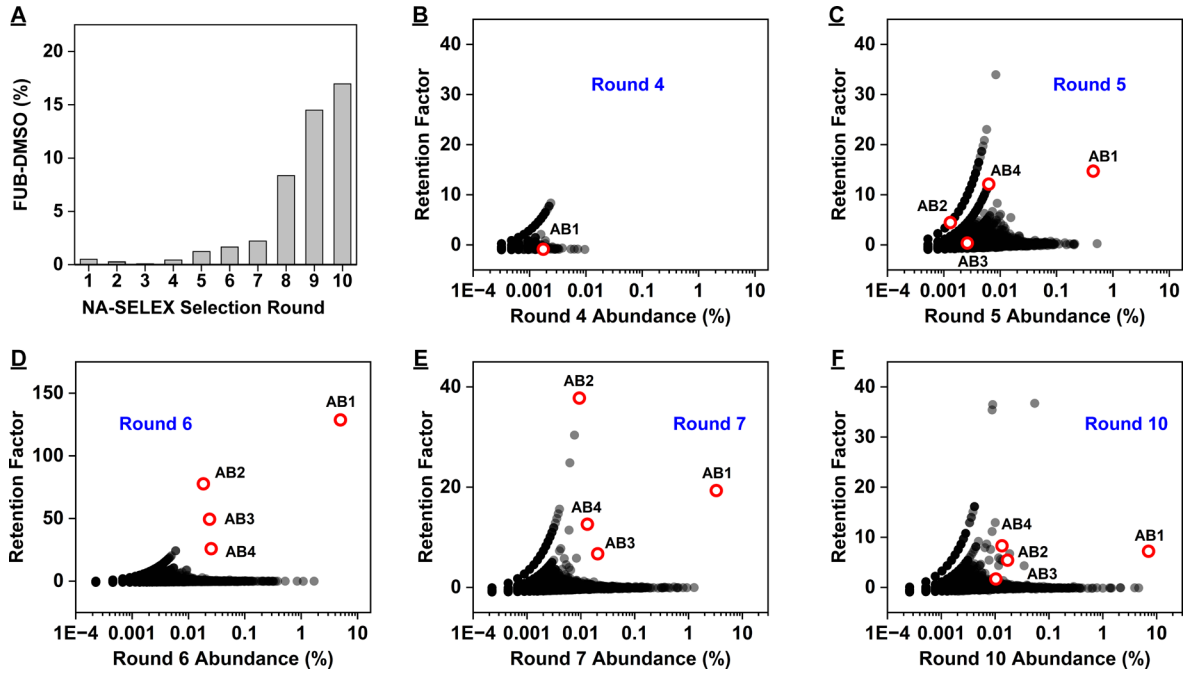

Figure 6. Progress of NA-SELEX over 10 rounds for AB-FUBINACA and HTS analysis of NA-SELEX pools. (A) The percentage difference of pool elution with versus without target over each round. (B–F) Retention factor plotted against abundance for each sequence in Rounds 4, 5, 6, 7, and 10 of NA-SELEX. Each data point represents a unique sequence, and aptamers AB1, AB2, AB3, and AB4 are indicated as red dots.

relatively rapidly for small-molecule targets that bind well at physiological temperatures and even more exceptionally under ambient conditions. This also indicated that the retention factor is an appropriate metric to identify binders. Both AB1 and AB2 are 83-nt in length and relatively G-rich, with guanine composing 17 of the 30 randomized nucleotides. To determine whether the primer-binding sites contribute to binding, we truncated these regions to generate AB1–53 and AB2–53 (Figure S4) and assessed their affinity with ITC. These truncated aptamers bound to AB-FUBINACA with similar affinities as their parent aptamers, with K_Ds of 31 ± 4 nM and 49 ± 4 nM at 23 °C and 444 ± 25 nM and 923 ± 51 nM at 37 °C for AB1-53 and AB2-53, respectively (Figure S5). In terms of the thermodynamics of binding, the ITC data indicated that aptamer-target binding is a largely enthalpy-driven process that occurs at the expense of a loss in entropy, which explains why affinity is improved at lower temperatures. To confirm that AB-FUBINACA specifically binds to these aptamers, and not any DNA oligonucleotide, we designed a point mutant of AB1-53 by mutating base 28 from G to T. According to both the exonuclease digestion assay and ITC, this mutant has no affinity for AB-FUBINACA (Figure S6). Finally, to assess the utility of AB1-53 and AB2-53 under physiological conditions, we determined their affinity for AB-FUBINACA in 1 × PBS with 1 mM $MgCl_2$. For AB1-53, we obtained K_Ds of 29 ± 6 nM and 1.0 ± 0.1 μM at 23 and 37 °C, respectively (Figure S7A), while for AB2-53, we obtained K_Ds of 54 ± 8 nM at 23 °C and 1.5 ± 0.1 μM at 37 °C (Figure S7B). Overall, the affinity of these aptamers does not meaningfully differ at room temperature when the concentration of Mg^{2+} is reduced from 5 mM to 1 mM and 1× PBS is employed, which suggests that the aptamers do not need excessive magnesium to function. However, at 37 °C, the affinity of the aptamers is reduced by roughly 2-fold, indicating that Mg^{2+} has some role in stabilizing the aptamer at higher temperatures.

Next, we determined the specificity of AB1-53 and AB2-53 to a wide range of potential interferent molecules. We employed a previously described assay in which aptamers are digested by a mixture of exonuclease III (Exo III) and exonuclease I (Exo I) with or without the target and various nontarget interferents, and the digestion process is monitored over time by sampling aliquots of the reaction mixture and staining with the fluorescent dye SYBR Gold.[19,20] In the absence of a ligand-binding event, the aptamer rapidly undergoes 3′-to-5′ exonuclease digestion into mononucleotides, but such digestion is greatly impeded when the aptamer is bound to a ligand. The magnitude of this resistance to digestion can be used as a proxy to estimate aptamer-ligand affinity. We digested AB1-53 and AB2-53 with Exo III and Exo I in the absence and presence of AB-FUBINACA and structurally similar interferents including serotonin, L-tryptophan, sumatriptan, dimethyltryptamine (DMT), lysergic acid diethylamide (LSD), tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabinol (CBN), as well as benzocaine, procaine, quinine, dehydroepiandrosterone sulfate (DHEAS), diphenhydramine, lidocaine, heroin, oxycodone, diazepam, alprazolam, cocaine, methylenedioxymethamphetamine (MDMA), amphetamine, methamphetamine, and methadone (Figure 5A). The assay indicated that these two aptamers were highly specific for AB-FUBINACA and did not yield any significant response to other ligands, either at room temperature or 37 °C (Figure 5B). This is even true for structurally related molecules such as serotonin, sumatriptan, and DMT, which contain an indole moiety (similar to the indazole of AB-FUBINACA), or natural cannabinoids THC, CBD, and CBN. These results indicate that the aptamers discovered through NA-SELEX not only have superior affinity, but excellent specificity as well, making them readily suitable for practical sensing applications.

To demonstrate the analytical utility of our aptamers, we used AB1 to detect AB-FUBINACA in human serum using a

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strand-displacement sensor.[12] To do so, we labeled AB1 with a 3′ Cy5 dye tag (AB1-Cy5), and modified a 14-nt complementary DNA with a 5′ Iowa Black RQ quencher group. Initially, the aptamer is hybridized with the cDNA, causing proximity-mediated quenching of Cy5. In the presence of target, the aptamer and cDNA dissociate, resulting in the recovery of fluorescence in a concentration-dependent manner. To optimize the assay, we first determined the concentration of cDNA required to achieve >80% quenching with a constant concentration of 50 nM AB1-Cy5, and found the concentration to be 250 nM (Figure S8A). Then, we challenged the aptamer-cDNA complex with 0−10 μM AB-FUBINACA in buffer, and observed a typical hyperbolic dose−response curve (Figure S8B), with signal saturation occurring at 1 μM, a linear range of 0−80 nM, and a limit of detection (LOD) of 20 nM (Figure S8C). From these measurements, we were also able to determine that AB1 bound to AB-FUBINACA with a K_D of 6 nM, which is similar to the ITC-reported value. We then challenged the fluorescence sensor with 50% filtered human serum spiked with AB-FUBINACA, and obtained nearly identical fluorescence recovery, linear range, and LOD values as in buffer (Figure S8B,C). Given the exceedingly low concentration of AB-FUBINACA in biological specimens (~5 nM), coupling this sensor with a signal amplification strategy such as enzyme-assisted target recycling[21] could enable the detection of this drug at clinically relevant levels.

Assessing the Reproducibility of NA-SELEX. Having demonstrated that NA-SELEX can efficiently isolate high-quality aptamers for small-molecule targets, we next determined the method's reproducibility. To do so, we carried out another trial of NA-SELEX with the same protocol and identical conditions using the same target one year after the initial successful trial. To avoid contamination from that trial, we used a newly synthesized library, cDNA, and primers as well as new buffers and reagents. Detailed selection conditions are provided in Table S6. Unlike in the previous trial, for the first five rounds, we did not observe any meaningful difference in pool retention (3−5%) when digestion was performed in the absence versus presence of target. However, in the sixth round, retention in the presence of target following digestion rose to 15.3%, relative to 13.6% in the absence of target (Figures S9 and S10). Following this sudden increase in pool resistance to digestion, we subjected the Round 5 and 6 pools to HTS. Surprisingly, we observed that AB1 was the most highly represented sequence in Round 5, with a relative abundance of 0.45% (Figure 6). Notably, when we plotted retention factor for Round 6 against Round 6 sequence abundance, AB1 clearly stood apart from other background sequences, with an abundance of 5% and retention factor of 128 (Figure S11). We also were now able to identify AB2, which had a retention factor of 78 and an abundance of 0.02%, as well as two new aptamers, AB3 and AB4, with a high retention factor (25−50) and moderate abundance (0.02−0.03%) (Figure S11). These aptamers were highly similar to AB1 in terms of sequence, and they belong to the same family (Figure S12). ITC showed that AB3 and AB4 respectively had K_D values of 20 ± 5 nM and 41 ± 6 nM at 23 °C and 471 ± 28 nM and 637 ± 27 nM at 37 °C (Figure S13). These results further confirm that NA-SELEX is a robust and reliable means of discovering high-quality aptamers for small-molecule targets.

Assessment of Pool Retention as a Viable Metric for Monitoring Aptamer Enrichment in NA-SELEX. Given that NA-SELEX is a new aptamer selection method, we next asked how future practitioners of NA-SELEX could reliably monitor aptamer enrichment during the selection process. In each round of NA-SELEX we performed for AB-FUBINACA, we had determined the proportion of pool retained after *Eco*RI digestion without versus with target. Logically, as NA-SELEX progresses and aptamers are enriched, pool retention in the presence of target should begin to exceed that of in the absence of target. However, since the proportion of aptamers in early rounds tends to be exceedingly low, it is questionable if pool retention measurements can be used to detect such a small difference in the pools with statistical significance. Thus, to determine the precision of pool retention measurements, we performed an experiment wherein we digested the naïve library or the Round 5 pool from the first trial of NA-SELEX four times independently with *Eco*RI in the absence and presence of AB-FUBINACA, and determined pool retention using PAGE analysis (Figure S14). We note that we did not perform negative selection prior to these digestion experiments, thus the pool retention values vary slightly from what was observed for SELEX. For the naïve library, we obtained pool retention values of 4.73 ± 0.35% and 4.97 ± 0.32% without and with target, respectively; according to an unpaired one-tailed *t* test, there is no significant difference between these values (p = 0.180). In contrast, for the Round 5 pool, we obtained pool retention values of 14.76 ± 0.61% and 15.95 ± 0.60% without and with target, respectively. An unpaired one-tailed *t* test confirms that pool retention in the presence of target is significantly higher than without target at a 95% confidence level (p = 0.016). These results indicate that when the population of binders is low—as is usually the case in the first few rounds of SELEX—and the precision of pool retention measurements is limited, pool retention is a poor metric for monitoring aptamer enrichment. Only when the population of binders becomes sufficiently high, as is observed in Round 5, can one use pool retention to monitor aptamer enrichment.

Realizing that is impractical to perform the same selection round multiple times to obtain statistically significant data, we next asked whether our HTS analysis approach was more feasible to monitor aptamer enrichment relative to using bulk pool retention values. To investigate this further, we returned to the Round 5 pool of the second trial of NA-SELEX and performed five more rounds, monitoring both bulk pool retention without and with target as well as examining the pools using HTS. We observed that the difference in pool retention with versus without target continued to increase, from a 1.2% difference in Round 5 to nearly 17% difference in Round 10 (Figure 6A). This indicates that target-specific differences in pool retention become more pronounced in the later stages of NA-SELEX. Ironically, however, as NA-SELEX progressed, from the perspective of HTS analysis, the retention factors for AB1, AB2, AB3, and AB4 decreased, such that by Round 10, AB2, AB3, and AB4 were nearly indistinguishable from the background (Figure 6B−F). This decrease in retention factor can be attributed to the buildup of background sequences, which went from 5% in Round 5 to 50% in Round 10. These data therefore indicate that, while performing more rounds of NA-SELEX might increase the difference in bulk pool retention with versus without target, doing so will obscure the presence of true aptamers when HTS analysis is performed. Hence, it is best to perform as few rounds of NA-SELEX as possible, monitor the abundance of sequences via HTS, and use retention factor to identify viable aptamer candidates.

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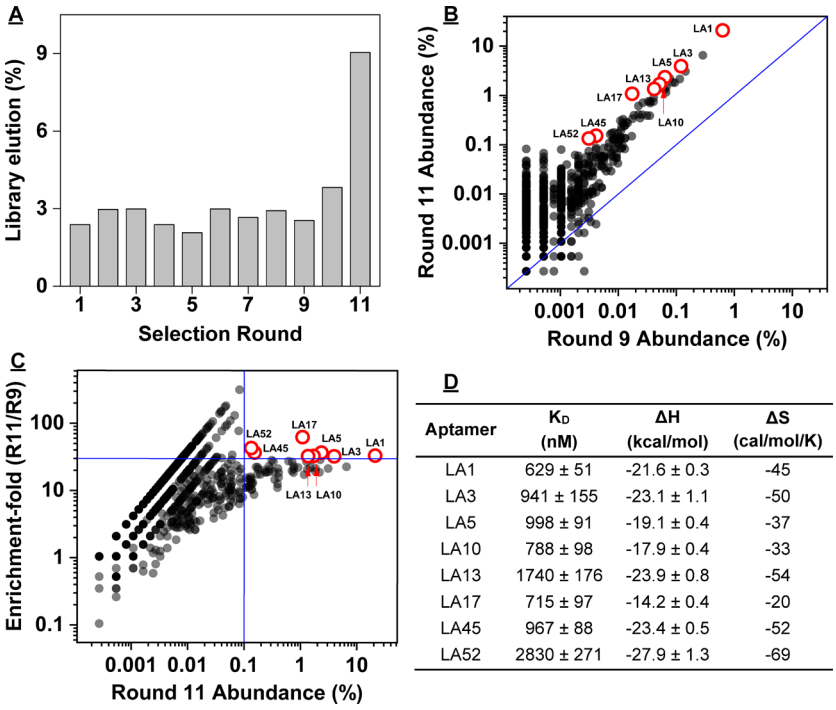

Aptamer	K_D (nM)	ΔH (kcal/mol)	ΔS (cal/mol/K)
LA1	629 ± 51	-21.6 ± 0.3	-45
LA3	941 ± 155	-23.1 ± 1.1	-50
LA5	998 ± 91	-19.1 ± 0.4	-37
LA10	788 ± 98	-17.9 ± 0.4	-33
LA13	1740 ± 176	-23.9 ± 0.8	-54
LA17	715 ± 97	-14.2 ± 0.4	-20
LA45	967 ± 88	-23.4 ± 0.5	-52
LA52	2830 ± 271	-27.9 ± 1.3	-69

Figure 7. Utilizing conventional library-immobilized SELEX to isolate aptamers that bind to AB-FUBINACA. (A) Elution of library by 100 μM AB-FUBINACA for every round of SELEX. (B, C) HTS analysis of the library-immobilized SELEX process. Each data point represents one sequence. Sequences with positive enrichment are above the blue diagonal line. For B, sequences are plotted based on their abundance in Round 11 versus their abundance in Round 9. In C, sequences are plotted based on their enrichment between Rounds 9 and 11 versus their abundance in Round 11. Sequences in red, which had enrichment fold >30 and abundance ≥0.1%, were chosen for further affinity characterization. (D) ITC-derived binding affinity, enthalpy, and entropy data for these aptamers at 23 °C.

Comparing the Efficiency and Performance of Library-Immobilized SELEX and NA-SELEX. Some targets are more amenable for aptamer generation than others due to their structure and physicochemical properties. For instance, the Stojanovic group recently reported that planar and aromatic targets—such as AB-FUBINACA—are more conducive to the isolation of high-affinity aptamers.[13] We therefore set out to determine whether library-immobilized SELEX[22] could yield aptamers as rapidly and with similar high quality as NA-SELEX. We began by hybridizing 0.6 nmol of the same library we used for NA-SELEX with a 5-fold excess of a 5′biotinylated version of cDNA15 (cDNA15-bio). We utilized the same selection buffer as in NA-SELEX and the same target concentration to ensure a fair comparison. Detailed selection conditions of library-immobilized SELEX are provided in Table S7. These library-cDNA complexes were immobilized onto streptavidin-coated agarose beads loaded in a microgravity column, and the beads were subsequently washed to remove library strands that weakly bound to the cDNA. We then added 100 μM AB-FUBINACA in three aliquots, collected the eluents, and amplified the isolated binders using PCR. In the following rounds, we employed lower quantities of library with the same target concentration (100 μM), and increased number of buffer washes to remove weakly bound library molecules. For the first nine rounds, we observed 2.5% elution on average by AB-FUBINACA, with target elution of pool consistently above pool elution with buffer alone. In Round 10, pool elution by the target rose to 3.8%, and increased again to 9.0% in Round 11 (Figure 7A).

We next subjected the Round 3, 4, 5, 9, and 11 pools to HTS. For Rounds 3, 4, 5, and 9, the proportion of unique sequences was ∼45%, indicating a lack of enrichment. However, the Round 11 pool consisted of 11% unique sequences, suggesting some degree of enrichment (Figure S15). Notably, whereas we were able to identify high-quality aptamers by Round 4 or 5 of NA-SELEX (Figure 4A), no such candidates were identified in the Round 3−5 pools from library-immobilized SELEX, with essentially all sequences exhibiting very low abundances of 0.001−0.004%. At this level of sequence abundance, the abundance readings are unreliable as they can have >100% error.[23] This demonstrates the greater efficiency of NA-SELEX relative to conventional selection methods. Only in Round 9 did we begin to observe the enrichment of certain sequences. The top-ranked sequence, LA1, had an abundance of 0.6%, and the top ten sequences made up 1.7% of the pool. In Round 11, the abundance of LA1 rose to 21% and now the top ten sequences made up 45% of the pool, which indicated that the pool was enriched (Figure 7B). We used ITC to characterize the affinity of all sequences (Figure S16) with an abundance ≥0.1% in Round 11 and an enrichment fold >30 between Rounds 9 and 11 (Figure 7C). Remarkably, these aptamers had 25−100-fold poorer affinity than the aptamers identified from NA-SELEX, with K_D ranging 0.6−2.8 μM (Figure 7D). The highest affinity aptamer, LA1, had a K_D of 629 nM at room temperature, which is 30-fold weaker than AB1 (K_D = 24 ± 3 nM), while aptamer LA52 had a K_D of 2.8 ± 0.27 μM. LA1 and its related sequences belong to a family (Figure S17) which is completely different from the AB aptamers. Most importantly, none of the aptamers identified by NA-SELEX nor any related family members were discovered in these library-immobilized SELEX pools. These results therefore indicated that NA-SELEX was more effective in yielding high-affinity aptamers relative to conventional approaches.

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Finally, we determined whether the "retention factor" metric we employed for NA-SELEX based on HTS data could identify aptamers in earlier rounds of library-immobilized SELEX. To do so, we reperformed Round 5 of library-immobilized SELEX, and, prior to adding the target to the library, we washed the immobilized library with 1× selection buffer and collected these eluents as the "negative sample". Then, we challenged the library with 100 μM AB-FUBINACA in buffer and collected these eluents as the "positive sample". We subjected both negative and positive samples to HTS, respectively obtaining 753,553 and 1,064,738 reads. Both samples consisted of 43% unique sequences (Figure S18A). Importantly, we did not find any of the LA aptamers we had discovered in Round 11 of library-immobilized SELEX in the positive sample. Only 0.0026% of the sequences (21 total) appeared in both negative and positive pools. To determine the retention factor for sequences that appeared in the positive pool but not the negative pool, we assigned the minimum reads per million value for negative pool abundance. We plotted the abundance of sequences in the positive pool against the library-immobilized SELEX analog of "retention factor" (i.e., positive pool abundance divided by negative pool abundance minus one). The abundance of sequences in the positive pool was very low (< 16 RPM), and no particular sequence stood out (Figure S18B). This data indicates that even when we used a similar HTS analysis strategy as we employed for NA-SELEX with library-immobilized SELEX, we could not identify any aptamers so early in library-immobilized SELEX. This is primarily because aptamers were not as enriched in library-immobilized SELEX by Round 5 relative to NA-SELEX.

CONCLUSION

We have developed a new nuclease-based aptamer selection technique termed NA-SELEX for the rapid discovery of DNA aptamers that bind small-molecule targets with high affinity and specificity at both room and physiological temperatures. NA-SELEX represents a major advancement in aptamer isolation methods for several reasons. First, we were able to isolate aptamers for a small molecule within just five rounds, which is similarly rapid as capillary electrophoresis SELEX approaches[24] and is demonstrably more rapid than library-immobilized SELEX. We performed a meta-analysis of 88 small-molecule aptamer selections via library-immobilized SELEX and found that the average number of rounds performed was 14 ± 4 with a median of 13 rounds (Table S1 and Figure S1). This is similar to the number of rounds we performed in this work using library-immobilized SELEX. We note one prior work that required only 3 rounds of selection,[25] but this utilized a library with low randomness (N8 random region, ~6.5×10^4 unique sequences) that is inherently highly enriched, which explains the rapidity of the selection process. In terms of selection time, a round of NA-SELEX takes 2 days to complete, while library-immobilized SELEX can take one to 2 days depending on the thoroughness of the counter-SELEX step. Nevertheless, NA-SELEX can yield aptamers in half the number of rounds with potentially superior binding performance. Second, another critical advantage of NA-SELEX is that since selection is performed by just incubating the library with nucleases, there is significantly less physical labor and "hands-on time" relative to conventional methods that require physical separation steps. In our own experience with library-immobilized SELEX, for example, this method requires continuous involvement and attention by the experimentalist for more than 8 h at a time. In theory, the convenience and simplicity of NA-SELEX could make it possible to perform multiple selections for different targets simultaneously, which could, in the long run, make NA-SELEX much more efficient compared to conventional selection methods. However, when performing multiplex selections, different libraries with different sets of primers should be used to avoid cross-contamination. Third, NA-SELEX yields aptamers that achieve effective binding at physiological temperatures, a major challenge for sequences obtained via room temperature selection processes.

To assess the reproducibility of NA-SELEX, we reperformed this selection for the same target using newly synthesized oligonucleotides for selection. We identified the same aptamers (AB1 and AB2) along with new ones from this second trial, demonstrating that the method is reproducible. It was surprising, however that two independent selection experiments could yield the same aptamer more than once, especially when considering we only utilized 10^{15} unique sequences in the starting library—equivalent to 0.1% of the theoretical sequence space (10^{18} unique sequences for a N30 library). Nevertheless, we have observed this same phenomenon in previous studies,[14,26] which suggests that synthetic oligonucleotide libraries only have a fraction of the diversity of the theoretical sequence space. This is reasonable, because not all sequences are likely synthesizable due to biases in reactivity or the insolubility or instability of certain oligonucleotides. In addition, it is possible that AB1 itself may have not been present in the initial library, but instead closely related sequences mutated into AB1 as a consequence of polymerase error during PCR. This is plausible, since we employed the relatively low-fidelity *Taq* polymerase,[27] and an oligonucleotide differing from AB1 by six bases is theoretically present in at least one copy per nanomole of N30 library.

Our investigation with *Eco*RI-based selection was first initiated more than five years ago. Recently, there has been a report of a nuclease-based technique that utilizes *Eco*RI to facilitate aptamer isolation in solution.[28] There are however several procedural differences between our NA-SELEX approach and the previously reported method, which consequently resulted in widely disparate outcomes. In particular, here using NA-SELEX, we identified aptamers in record time (5 rounds) with nanomolar affinity, whereas in the previous report, more than ten rounds were required to obtain aptamers, which had a K_D of 300−400 μM—a nearly 10,000-fold difference in affinity. The first notable difference in our methodology is how the library is recovered following negative selection. Here, we used Klenow fragment to regenerate our library, which had consistently high recoveries of >90%, while the previous report utilized DNA Ligase I with a lower efficiency of 50%. Second, after positive selection and prior to PCR, we performed an additional PAGE purification step to remove cleaved sequences, whereas the previously reported approach did not perform this step. We have found in previous failed trials of NA-SELEX that the carryover of cleaved sequences to the PCR stage greatly depletes the reverse primer, especially in later rounds of SELEX, thereby significantly hampering the amplification of true binders, lowering PCR efficiency, and perhaps delaying the enrichment of aptamers. This is possibly evidenced by the 99.9% sequence diversity of the Round 4 pool in the previous report, relative to our more extensively enriched Round 4 pool with 24% sequence

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diversity. One final important difference is the way in which we analyzed the HTS data sets to identify aptamer candidates. In the previous report, aptamer candidates were chosen based on their enrichment fold above a certain threshold between the last few rounds of selection. In contrast, we selected candidates based on their abundance when pools were digested without versus with target. By using this metric (which we termed here retention factor), we were able to easily identify binding sequences.

We foresee the opportunity to customize many aspects of NA-SELEX to achieve specific goals. For instance, the design of the library can be altered in terms of the length and even the nucleotide composition of the random domain, so as long as the restriction site is installed on each library strand. NA-SELEX based on *Eco*RI can also be performed at room temperature[29] or with varying ionic strength (e.g., 1−10 mM $MgCl_2$).[30] In addition, Counter-SELEX can be easily adapted into NA-SELEX. In particular, during the negative selection step, interferent(s) can be incubated with the library, followed by digestion with *Eco*RI. Any sequences that bind an interferent will dissociate from the cDNA and thus avoid *Eco*RI cleavage, while sequences that do not bind the interferent remain bound to the cDNA and are cleaved by *Eco*RI. The cleaved library strands are then recovered, elongated with Klenow, and subjected to positive selection. We also predict it is highly feasible that other restriction enzymes could be employed to facilitate NA-SELEX. One potential upgrade to *Eco*RI is converting to a nicking endonuclease such as Nt.BbvCI, which cleaves only one strand of DNA instead of both.[31] This could allow cleavage of the library strand only, thereby preserving the cDNA during selection.

Since NA-SELEX and library-immobilized SELEX share the same target-induced strand-displacement selection mechanism, we hypothesize that NA-SELEX is just as generalizable as library-immobilized SELEX. However, this remains to be proven empirically. We see no chemical reason why NA-SELEX would not work for other small molecules, or even proteins for that matter. However, we do anticipate two potential problems. The first is targets that directly inhibit *Eco*RI—although we are currently unaware of any molecules that do this—or that sequester its cofactor, Mg^{2+}. Another concern is that background resistance to enzymatic digestion seems to spike at the fifth or sixth round. If there are targets for which the number of binders in the library is too small, it may be necessary to perform a few pre-enrichment rounds using a conventional method like library-immobilized SELEX. After this pre-enrichment step, we predict that NA-SELEX should be able to efficiently enrich target-binding aptamers in no more than three or four rounds. While not purely NA-SELEX, the process could still prove more expedient than library-immobilized SELEX alone, with a greater likelihood of identifying high-quality aptamers. With regard to rising background levels during NA-SELEX, we hypothesize that resistant sequences are those that have a melting temperature of ∼37 °C when complexed with the cDNA. Such sequences simultaneously coexist in a cDNA-bound and unbound form during selection, and could therefore bypass negative selection and also survive positive selection without being able to bind the target. If aptamers are not identified before resistant sequences overtake the pool, it is best to first perform a pre-enrichment step using another SELEX method to increase the population of binders prior to NA-SELEX. Although, as noted above, the success rate of traditional aptamer selection methods for small-molecule targets is highly dependent on the structure and physicochemical characteristics of those targets, we believe that NA-SELEX could offer an effective strategy for accelerating the selection process and yielding superior quality aptamers to those generated via existing techniques.

MATERIALS AND METHODS

NA-SELEX Protocol. Detailed information for the concentrations of library, cDNA, and target used for each round of NA-SELEX is provided in the Supporting Information. Each round consists of three main steps: negative selection, elongation of cleaved library with Klenow fragment, and positive selection.

Negative Selection. Negative selection was first performed to remove library sequences that inherently resist *Eco*RI digestion. The library (LibeB3) was mixed with a 5-fold excess of cDNA15 in 0.5× PBS (pH 7.4). The mixture was heated in boiling water for 10 min and cooled to room temperature in a water bath for 20 min. Then, $MgCl_2$, DTT and Triton X-100 were added to reach a final concentration of 5 mM, 1 mM and 0.005% (v/v), respectively. Next, 86 μL of this solution was aliquoted into several PCR tubes and mixed with 4 μL DMSO and incubated at 37 °C for 1 h. An aliquot was taken and added to loading buffer as a reference sample for the predigestion library. To initiate the enzymatic reaction, 10 μL *Eco*RI-HF was added (final concentration 0.2 U/μL). After digestion for 2.5 h, *Eco*RI-HF was deactivated by heating the sample at 65 °C for 20 min. An aliquot of this solution was taken and mixed with loading buffer for characterization of digestion efficiency by PAGE. The digestion product was concentrated with a 500 μL 3 kDa filter by centrifuging at 15,000 rcf for 6 min, and then buffer and salts were removed by washing twice using molecular biology-grade water. The concentrated product was completely dried using an Eppendorf Vacufuge Plus vacuum centrifuge and then reconstituted in 12 μL PAGE purification loading buffer. The digestion product was loaded into a 12% urea-denatured polyacrylamide gel and run at 100 V for 20 min and then 450 V for 90 min in 0.5× TBE running buffer heated to 65 °C. The gel was then illuminated with a UV lamp (λ = 254 nm), and bands containing the cleaved library were excised and combined. The excised gel was crushed, mixed with 1× Tris-EDTA buffer (1 part gel to 6 parts buffer), and incubated at 65 °C for 3 h to allow the DNA to elute. Afterwards, the supernatant was placed in a 500 μL 3 kDa filter and concentrated as above, and the concentrate was washed three times with water to remove salts and contaminants. The DNA concentration was measured using a NanoDrop 2000 spectrophotometer. Finally, all collected reference samples were analyzed by 15% urea-denatured PAGE to assess the negative selection process.

Restoration of Library. For the elongation of the cleaved library using Klenow fragment, all cleaved library was annealed with a 2.5-fold higher quantity of 30-nt 5′ biotinylated splint (splint-bio) in NEBuffer 2 (final concentrations: 10 mM Tris-HCl buffer, pH 7.9 at 25 °C, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) at a final volume of 100 μL via heating at 95 °C in boiling water for 10 min and then cooling in a water bath to room temperature for 20 min. Afterwards, deoxynucleotide triphosphate (dNTP) solution was added (final concentration 33 μM) and mixed, and an aliquot was taken and added to loading buffer as a reference sample for the pre-elongated library. Then, Klenow fragment (final concentration 0.02 U/μL) was added to initiate elongation. The solution was incubated at 25 °C for 15 min. The polymerase was inactivated by adding EDTA (final concentration 10 mM) and heating at 75 °C for 20 min. An aliquot was taken before and after enzyme inactivation and added to loading buffer as a reference sample for the restored library. The solution was flowed through a microcolumn containing 250 μL streptavidin-immobilized agarose resin prewashed with 1× PBS three times to ensure that the oligonucleotides were immobilized. The column was washed three times with 250 μL of 1× PBS to remove salts, dNTPs, and enzyme. To elute the library, the column was capped, 500 μL 0.2 M NaOH was added and incubated for 10 min, and then the eluent was

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collected and neutralized with 1 M hydrochloric acid (HCl). This solution was then added to a 500 μL 3 kDa filter and washed with water three times to remove salts. An aliquot was taken from the library and added to loading buffer as a reference sample. DNA concentrations were measured using a NanoDrop 2000 spectrophotometer. All collected reference samples were analyzed by 15% urea-denatured PAGE to assess the library recovery process.

To amplify the restored library for positive selection, from the second round, ∼30% of the recovered library (∼65 pmol) was used as a template for PCR with 1 μM forward primer (FP) and 1 μM biotinylated reverse primer (RP-bio) in 1 mL 1× Hot-Start GoTaq Colorless PCR Master Mix. PCR was performed using a BioRad C1000 thermal cycler with the following conditions: 2 min at 95 °C; 9 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 45 s, followed by 5 min at 72 °C. After PCR amplification, the amplicons were combined and subjected to single-stranded DNA generation. Specifically, the amplicon solution was added to a column containing 200 μL of streptavidin-immobilized agarose beads prewashed with 1× PBS. The solution was added to the column three times to ensure library immobilization. Then, the column was washed with 250 μL of 1× PBS six times to remove dNTPs, salts, and enzyme. The column was capped and 400 μL of 0.2 M NaOH was added and incubated for 10 min. The eluent was then collected, neutralized with 1 M HCl, and desalted with water using a 500 μL 3 kDa filter. DNA concentrations were determined using a NanoDrop 2000 spectrophotometer.

Positive Selection. This step was performed to enrich sequences binding to AB-FUBINACA. First, the elongated library (final concentration: 2 μM) was mixed with a 5-fold higher quantity of cDNA in 0.5× PBS (pH 7.4). The annealing procedure was performed as described above, and then $MgCl_2$, DTT and Triton X-100 were added (final concentrations are 5 mM $MgCl_2$, 1 mM, and 0.005% (v/v), respectively). This mixture was divided into two PCR tubes with a volume of 86 μL each. For control and positive samples, 4 μL of DMSO or AB-FUBINACA (final concentration 100 μM) were added to the solution, respectively. The samples were incubated at 37 °C for 1 h, and then 10 μL of *Eco*RI-HF was added (final concentration 0.2 U/μL). Digestion time was optimized to minimize the retention of binding-incompetent strands while maximizing the retention of target-binding aptamers. To estimate optimal digestion time, we digested the naïve library with *Eco*RI-HF in the absence of target and observed that the lowest level of library retention (∼2−3%) was obtained after 5 h of digestion. Therefore, for all rounds, digestion was performed at 37 °C for 5 h. Afterward, *Eco*RI-HF was deactivated by heating at 65 °C for 20 min. An aliquot was taken before enzyme addition and after enzyme inactivation and added to loading buffer as a reference sample. The digestion product was concentrated, washed with water, dried, reconstituted in PAGE purification loading buffer, and run on a 12% urea-denatured polyacrylamide gel as described above. Afterwards, the gel was illuminated with a UV lamp and bands containing the full-length library from each sample were excised and recovered. The DNA was eluted from the gel using the crush-and-soak method and purified as described above. The library was PCR amplified as explained above but with the following conditions: 2 min at 95 °C; 11 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 45 s, followed by 5 min at 72 °C. After PCR amplification, the amplicons were subjected to single-stranded DNA generation as explained above. DNA concentrations were determined using a NanoDrop 2000 spectrophotometer. All collected reference samples were analyzed by 15% urea-denatured PAGE to assess the library recovery process.

High-Throughput DNA Sequencing (HTS) and Data Analysis. Pools from *in vitro* selection (5 pmol) were PCR amplified with primers containing partial Illumina adapters (FP-HTS and RP-HTS, 1 μM each) in 50 μL 1× Hot Start Colorless Master Mix using a BioRad C1000 thermal cycler with the following conditions: 2 min at 95 °C; 11 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 45 s, followed by 5 min at 72 °C. The amplicons were then purified with a PCR purification kit (Qiagen). PAGE was performed to confirm successful PCR amplification and purification. The amplicons were sequenced by Genewiz LLC using an Illumina sequencing platform. For each fastq file, the primers were first trimmed using cutadapt software (version 4.4),[32] after which sequences were counted using fastaptamer (version 1.0.3).[33] The total reads and sequence abundance in each round from NA-SELEX trial 1 and trial 2 as well as library-immobilized SELEX is provided in Tables S8−13. Sequence logos were generated using the web-based application WebLogo.[34] Retention factor was calculated using the following equation: $(P - P_0)/P_0$, where P is the population of a particular sequence in the target-selection pool and P_0 is the population of that sequence in the control pool. To calculate retention factor if a sequence appeared in the positive pool but not in the control pool, that sequence was assigned an abundance equivalent to the minimum reads per million value for that round's control (negative) pool. For example, AB2 had an abundance of 0.038% in the Round 5 positive pool but was not identified in the Round 5 negative pool. Therefore, AB2 was assigned a negative pool abundance of 0.00055% (equivalent to one read in that pool) to calculate retention factor. Graphs of sequence abundance plotted as a function of retention factor only include sequences that were detected in the positive pool for that respective round.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.4c00748.

Additional and materials and methods used for this paper, a table containing information on previous library-immobilized SELEX experiments in the literature, list of DNA sequences used in this work, selection strategy and conditions for NA-SELEX, statistics for HTS data sets, population of most abundant sequences in NA-SELEX, selection conditions for library-immobilized SELEX, the total reads and sequence abundance in each round from NA-SELEX trial 1 and trial 2 as well as library-immobilized SELEX, summary of ITC data, a histogram for number of rounds performed for library-immobilized SELEX to isolate small-molecule binding aptamers, proportion of unique sequences in SELEX pools, *Eco*RI digestion of aptamers isolated via NA-SELEX, secondary structures of AB1-53 and its single point mutant as well as AB2-53, ITC data for binding of AB1 and AB2 to their target, strand-displacement sensor for AB-FUBINACA detection, PAGE data and summary for the second trial of NA-SELEX, analysis of HTS data for second trial of NA-SELEX, sequence logo of AB family, ITC data for AB3 and AB4 binding to their target, proportion of unique sequences in library-immobilized SELEX, and sequence logo and secondary structures for LA1 aptamer family (PDF)

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Notes

The authors declare no competing financial interest.

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