

# Reverse Transcription of Threose Nucleic Acid by a Naturally Occurring DNA Polymerase

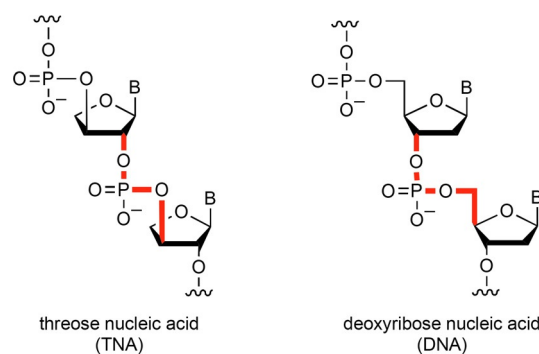
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Recent advances in polymerase engineering have enabled the replication of xenonucleic acid (XNA) polymers with backbone structures distinct from those found in nature. By introducing a selective amplification step into the replication cycle, functional XNA molecules have been isolated by in vitro selection with binding and catalytic activity. Despite these successes, coding and decoding genetic information in XNA polymers remains limited by the fidelity and catalytic efficiency of engineered XNA polymerases. In particular, the process of reverse transcribing XNA back into DNA for amplification by PCR has been problematic. Here, we show that *Geobacillus stearothermophilus* (Bst) DNA polymerase I functions as an efficient and faithful threose nucleic acid (TNA)-dependent DNA polymerase. Bst DNA polymerase generates ~twofold more cDNA with threefold fewer mutations than Superscript II (SSII), which was previously the best TNA reverse transcriptase. Notably, Bst also functions under standard magnesium-dependent conditions, whereas SSII requires manganese ions to relax the enzyme's substrate specificity. We further demonstrate that Bst DNA polymerase can support the in vitro selection of TNA aptamers by evolving a TNA aptamer to human  $\alpha$ -thrombin.

Recent advances in polymerase engineering have enabled the replication of a wide range of xenonucleic acid (XNA) polymers with backbone structures that are unique relative to those found in natural DNA and RNA.<sup>[1–3]</sup> Although such systems have been used to evolve XNA molecules with ligand binding and catalytic activities,<sup>[4–6]</sup> the current generation of engineered polymerases function with reduced activity relative to natural polymerases.<sup>[7,8]</sup> The development of XNA polymerases with strong reverse transcriptase activity, in particular, has posed a significant bottleneck in the in vitro replication of XNA polymers. Although considerable progress has been made in the development of DNA-dependent XNA polymerases (also known as XNA synthases),<sup>[9]</sup> methodologies developed to engineer these enzymes are often restricted to DNA-templated reactions.<sup>[6,10,11]</sup> Consequently, most XNA reverse transcriptases have been identified by screening polymerases for DNA synthesis activity on an XNA template rather than by directed evolution.<sup>[6,12–14]</sup>

Previously, we showed that an engineered version of a reverse transcriptase isolated from the Moloney murine leukemia

virus, which is sold commercially under the trade name Superscript II (SSII), can function as a reasonably efficient threose nucleic acid (TNA)-dependent DNA polymerase.<sup>[12,13]</sup> TNA is an artificial genetic polymer in which the natural ribose sugar found in RNA has been replaced with an unnatural four-carbon threose sugar (Scheme 1). The observation that SSII could reverse transcribe TNA into DNA was attributed to the A-like helical geometry of the TNA/DNA duplex,<sup>[15]</sup> which closely approximates the natural helical geometry of DNA/RNA hybrids. When used in combination with Kod-RI, an engineered TNA polymerase created by combinatorial library screening and scaffold sampling, the aggregate fidelity for a complete replication cycle of DNA  $\rightarrow$  TNA  $\rightarrow$  DNA is  $\sim 1.5 \times 10^{-2}$ , which is slightly



**Scheme 1.** Constitutional structure for the linearized backbone of threose nucleic acid (TNA; left) and DNA (right). TNA is an unnatural genetic polymer composed of repeating  $\alpha$ -L-threose sugars vicinally connected by 2',3'-phosphodiester bonds. Differences in backbone linkages are highlighted in red.

higher than one misincorporation per 100 nucleotides.<sup>[16]</sup>

In an effort to improve the fidelity of TNA replication, we re-examined a previous screen of commercial polymerases that were tested for the ability to copy TNA into DNA.<sup>[12]</sup> In that study, the *Geobacillus stearothermophilus* DNA polymerase I (Bst) large fragment was identified as one of a small number of DNA polymerases that were capable of extending a DNA primer annealed to a short synthetic TNA template with dNTP substrates. More recently, Bst was shown to transcribe and reverse transcribe limited stretches of glycerol nucleic acid (GNA) polymers,<sup>[17,18]</sup> as well as copy RNA templates into DNA,<sup>[19]</sup> suggesting that this enzyme could have broader template specificity than is typical of most naturally occurring DNA polymerases.

We began by examining the ability of Bst DNA polymerase to copy a library of TNA templates into DNA. We chose to monitor the polymerase extension reaction by denaturing polyacrylamide gel electrophoresis (PAGE), which makes it pos-

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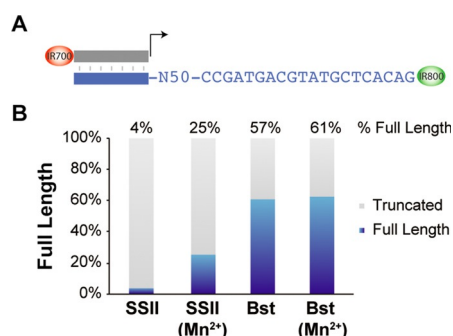
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sible to measure the fraction of primer that is extended to full-length product and the level of truncated products that accumulate during the course of the reaction. A library of TNA molecules was constructed by copying a DNA library into TNA using Kod-RI and a synthetic DNA primer carrying an IR800 dye at the 5'-end. This reaction produced a library of IR-labeled TNA templates that were uniform in length (90 nt) but carried an internal random region of 50 unbiased nucleotide positions. Bst and SSII were then challenged to copy the TNA library back into DNA by extending a 5'-IR700 labeled-DNA primer with dNTP substrates (Figure 1A). Both reactions were performed at their optimal reaction temperatures (42 °C for SSII and 55 °C for Bst) in their preferred commercial buffers (First Strand buffer [50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl<sub>2</sub>, pH 8.3] and Thermopol buffer [20 mM Tris-HCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 mM KCl, 2 mM MgSO<sub>4</sub>, 0.1% Triton X-100, pH 8.8], respectively). Reactions were performed in the presence and absence of MnCl<sub>2</sub>, which is known to improve polymerase incorporation of non-cognate substrates by lowering substrate specificity.<sup>[20, 21]</sup>

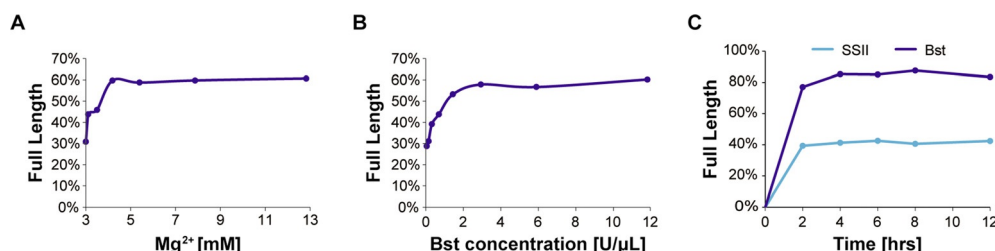
Consistent with previous reports, SSII was found to generate modest amounts of full-length DNA product in the presence of manganese ions but failed to extend the primer in the absence of MnCl<sub>2</sub> (Figure 1B, Figure S1 in the Supporting Information).<sup>[13]</sup> In contrast, Bst produced nearly equivalent amounts of

full-length DNA product, regardless of whether MnCl<sub>2</sub> was present or absent from the reaction mixture (Figure 1B). In total, Bst extended ~60% of the DNA primer into full-length cDNA product, whereas SSII supplemented with manganese ions extended only ~25% of the primer to full-length product. This striking difference demonstrated that, within the context of TNA reverse transcription, Bst DNA polymerase functions with higher primer extension efficiency and lower sequence bias than SSII. To improve the efficiency of cDNA synthesis, we determined the magnesium ion dependency and polymerase concentration required for optimal TNA-dependent DNA synthesis by Bst DNA polymerase. By gradually supplementing the reaction buffer with MgCl<sub>2</sub> or increasing the enzyme concentration, we found that Bst functioned with optimal activity at 6 mM MgCl<sub>2</sub> and an enzyme concentration of 1.6 U μL<sup>-1</sup> (Figure 2A, B). Under each polymerase's optimal conditions, a time-course analysis revealed that Bst DNA polymerase converted ~80% of the starting primer into full-length cDNA product, whereas SSII supplemented with manganese ions achieved only ~40% product conversion (Figure 2C). In both cases, after an initial burst, the reaction profile reaches a plateau after 2 h of incubation; this suggests that longer incubation times are unlikely to yield higher amounts of elongated product. Analysis of the gel revealed that SSII generated noticeable amounts of truncated product, as indicated by a growing smear beneath the full-length product band (Figure S1). The time-course reactions performed under optimal conditions mirrored our initial data and further supported the observation that Bst DNA polymerase functions with higher primer-extension efficiency and lower sequence bias than SSII.

To ensure that the improved activity associated with Bst DNA polymerase did not come at a negative cost to polymerase fidelity, we measured the aggregate fidelity for TNA replication. This term refers to the fidelity observed when a DNA template is taken through a complete replication cycle (DNA → TNA → DNA), which is operationally different than the more restricted view of fidelity as a single-nucleotide incorporation event. Aggregate fidelity is an important parameter of polymerase function, as it reflects the combined effects of nucleotide misincorporations, insertions, and deletions, as well as the sequence context in which these mistakes occur.<sup>[6, 8, 9, 13, 16]</sup> Accordingly, a DNA template of known sequence is transcribed into TNA by using Kod-RI, PAGE purified, reverse transcribed



**Figure 1.** SSII and Bst-mediated synthesis of cDNA on a library of TNA templates. A) Schematic representation of a TNA reverse transcription reaction. An IR-labeled DNA primer (gray) was annealed to a naïve TNA library (blue) and extended with dNTPs. N denotes a random region containing all four nucleobases. B) The amount of full-length cDNA product generated by Bst and SSII DNA polymerases. Primer-extension reactions were performed for 1 h under standard reaction conditions in the presence or absence of MnCl<sub>2</sub>. Full-length data derived from the gel are shown in Figure S1.



**Figure 2.** Bst-mediated TNA reverse transcription. Bst-mediated reverse transcription reactions were optimized for A) magnesium ion dependency and B) enzyme concentration. Primer-extension reactions were performed for 1 h at 55 °C on a random library of TNA templates. C) Time-course analysis comparing the amount of full-length cDNA product obtained by SSII and Bst DNA polymerases. Primer-extension reactions were performed under the optimal reactions and the temperature determined for SSII and Bst. Full-length data derived from gels are shown in Figure S2.

back into cDNA, amplified by PCR, cloned, and sequenced (Figure S3).

To ensure that the cloned sequence came from the TNA replication cycle, reverse transcription controls are performed (Figure S4), along with a mismatched forward primer that produces a single-nucleotide transversion in the reverse transcribed cDNA product (Figure S3). Only cDNA products carrying the single-nucleotide transversion are analyzed for fidelity.

In total, we compared the aggregate fidelity for TNA replication experiments performed with SSII supplemented with manganese ions and Bst DNA polymerase tested in the presence and absence of manganese ions (Table 1). For reactions performed with  $\text{MnCl}_2$ , SSII and Bst yielded mutation rates of 20 and 163 mistakes per 1000 nucleotide incorporations, respectively. However, in the absence of  $\text{MnCl}_2$ , Bst had an error rate of only four mistakes per 1000 nucleotides, which is 43 times lower than the manganese-supplemented reaction and five times lower than SSII.

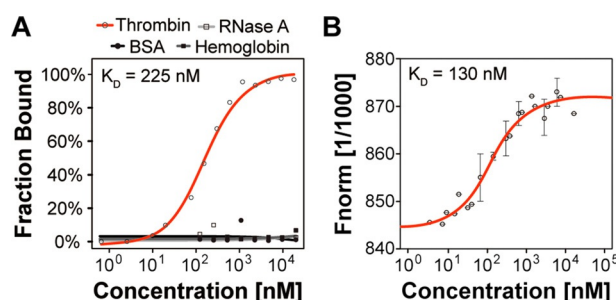
**Table 1.** Aggregate fidelity for TNA replication with SSII and Bst reverse transcriptases.

	SSII	Bst	Bst
$\text{MnCl}_2$	+	+	–
insertions	0	21	0
deletions	2	55	2
substitutions	33	46	2
base reads	1698	746	1048
mutation rate ( $\times 10^{-3}$ )	20.6	163.5	3.8

To test the fecundity of the Bst and Kod-RI polymerase pair, we subjected a random library of  $10^{10}$  unique TNA molecules to iterative rounds of in vitro selection and amplification. For each round of selection, a library of DNA templates was transcribed into TNA by extending a fluorescein-labeled DNA primer–template complex with tNTP substrates. The pool of TNA molecules was gel purified and incubated with human  $\alpha$ -thrombin in selection buffer (10 mM Tris, 100 mM NaCl, 1 mM  $\text{MgCl}_2$ , pH 8.3). Functional molecules that bound the protein target were isolated by capillary electrophoresis (CE; Figure S5).<sup>[22–25]</sup> We chose CE as the partitioning technique, because it provides a solution-based approach for separating bound aptamers from the unbound pool, which helps reduce the occurrence of non-specific binders commonly present in traditional bead-based selections. The TNA–protein mixture was injected into an eCAP neutral capillary (50  $\mu\text{m}$  i.d., 60 cm long) and electrophoresed at 30 kV. Under these conditions, the aptamer–thrombin complex migrated faster than the unbound TNA pool, which enabled us to collect and recover the bound TNA–protein complex (Figure S6). The TNA from each round of selection was reverse transcribed by Bst and amplified by PCR. After three rounds of in vitro selection and amplification, the library was cloned and sequenced.

We chose ten random isolates for affinity binding to human  $\alpha$ -thrombin. Isolates were transcribed into TNA with a fluorescently labeled DNA primer. One aptamer sequence, T10 did

not transcribe to full-length and was discarded. The other nine aptamers were gel purified and tested for solution binding affinity by using a membrane-binding dot blot assay. In this strategy, a nitrocellulose membrane selectively captures the unbound protein and bound protein–aptamer complexes, whereas free aptamers pass through the nitrocellulose membrane and are captured on the underlying nylon membrane.<sup>[5,26]</sup> The binding affinities of the nine TNA aptamers were in the range of  $\sim 200$ – $1000$  nM (Table S1). TNA aptamer T1 provided the tightest binding affinity to human  $\alpha$ -thrombin with a solution dissociation constant ( $K_D$ ) of 225 nM (Figure 3A). Additionally, T1 showed high specificity against BSA, hemoglobin, and RNase A (Figure 3A).



**Figure 3.** Characterization of a TNA aptamer identified by in vitro selection. A) Solution binding affinity of aptamer T1 against human  $\alpha$ -thrombin, BSA, hemoglobin, and RNase A by using membrane-binding analysis. B) Validation of aptamer T1 binding affinity to human  $\alpha$ -thrombin by using microscale thermophoresis. Data represents the average of five trials.

We confirmed the  $K_D$  value of T1 binding to human  $\alpha$ -thrombin by using microscale thermophoresis (MST).<sup>[27–29]</sup> MST is a biophysical method in which binding affinities are measured in solution by monitoring the movement of particles in a microscopic temperature gradient. Differences in the hydrodynamic shell between aptamer–protein complexes and free aptamer result in a relative change of movement along the temperature gradient. By using MST, aptamer T1 was found to bind human  $\alpha$ -thrombin with a  $K_D$  value of 130 nM, as determined by the average of five independent replicates (Figure 3B). The strong correlation between the affinities measured by dot blot and MST suggest that the  $K_D$  value of aptamer T1 is  $\sim 200$  nM. Furthermore, aptamer T1 retained activity in the presence of RNase A, whereas binding of an RNA aptamer selected by using the same strategy was lost (Figures S7 and S8, Table S2). This finding is consistent with previous studies demonstrating that short stretches of TNA are highly resistant to nuclease degradation.<sup>[13,30]</sup>

In summary, we demonstrated that Bst DNA polymerase is an efficient and faithful TNA-dependent DNA polymerase with activity superior to that of SSII. We further showed that a TNA replication system composed of Bst and Kod-RI can be used to support the in vitro selection of TNA aptamers. This is the first demonstration in which a two-enzyme replication system was used to isolate a TNA aptamer by in vitro selection. In contrast with our previous DNA display approach,<sup>[5]</sup> the two-enzyme replication system should make it easier to evolve TNA mole-

cules with more complicated functions, like catalysis. Additionally, we suggest that Bst DNA polymerase provides a starting point for evolving TNA and other XNA reverse transcriptases that can be used to assemble a xenobiology toolkit.

## Experimental Section

**Generation of DNA template for TNA transcription:** Single-stranded DNA templates were generated by PCR. Each PCR reaction (1000  $\mu\text{L}$  total volume) contained each DNA primer (PBS7 PEG Long and PBS8; 1000 pmol) and double-stranded DNA template (50 pmol). Reactions were performed in 1 $\times$  ThermoPol buffer (20 mM Tris-HCl, 10 mM  $(\text{NH}_4)_2\text{SO}_4$ , 10 mM KCl, 2 mM  $\text{MgSO}_4$ , 0.1% Triton X-100, pH 8.8) supplemented with dNTPs (400 nM), and DV *exo*- (0.04  $\text{U } \mu\text{L}^{-1}$ ). The solution was divided into 50  $\mu\text{L}$  reaction volumes and underwent the following steps on a thermocycler: 2 min at 95  $^\circ\text{C}$ , followed by 15 cycles of 15 s at 95  $^\circ\text{C}$ , 15 s at 58  $^\circ\text{C}$ , and 2.25 min at 72  $^\circ\text{C}$ . The reactions were combined, concentrated by lyophilization, and supplemented with 5 mM EDTA and 50% (w/v) urea. The solution was heat-denatured for 5 min at 95  $^\circ\text{C}$ , and the PEG-modified strand was purified by 10% denaturing urea PAGE. The corresponding band was excised, electroeluted, and concentrated by ethanol precipitation. The precipitated pellet was resuspended in nuclease-free water (20  $\mu\text{L}$ ) and quantified by UV absorbance.

**TNA transcription:** The TNA template was generated in a single 100  $\mu\text{L}$  reaction volume containing DNA primer-template complex (50 pmol). The complex was labeled at the 5'-end of the DNA primer with IR800 dye. The primer-template complex was annealed in 1 $\times$  ThermoPol buffer (20 mM Tris-HCl, 10 mM  $(\text{NH}_4)_2\text{SO}_4$ , 10 mM KCl, 2 mM  $\text{MgSO}_4$ , 0.1% Triton X-100, pH 8.8) by heating for 5 min at 95  $^\circ\text{C}$  and cooling for 10 min at 4  $^\circ\text{C}$ . Kod-RI TNA polymerase (10  $\mu\text{L}$ ) was pretreated with  $\text{MnCl}_2$  (1 mM), then added to the reaction mixture. Nucleotide triphosphates (100  $\mu\text{M}$ ) were added to the reaction, and the solution was incubated for 4 h at 55  $^\circ\text{C}$ . The reaction was treated with proteinase K (10  $\mu\text{L}$ ) for 1 h at 55  $^\circ\text{C}$ , quenched by adding 50% (w/v) urea and a final concentration of 10 mM EDTA, and heat-denatured for 5 min at 95  $^\circ\text{C}$ . TNA was purified by 20% denaturing PAGE, electroeluted, concentrated on a YM-10 microcentrifuge column, and quantified by UV absorbance according to Beer's law.

**TNA reverse transcription:** Reverse transcription reactions were performed in a 10  $\mu\text{L}$  reaction volume. For SSII reactions, PBS1 IR700 primer (5 pmol) was annealed to TNA template (10 pmol) in 1 $\times$ First Strand Buffer (50 mM; Tris-HCl, 75 mM KCl, 3 mM  $\text{MgCl}_2$ , pH 8.3) by heating for 5 min at 95  $^\circ\text{C}$  and cooling for 10 min at 4  $^\circ\text{C}$ . dNTPs (500  $\mu\text{M}$ ) and DTT (10 mM) were added to the primer template complex, and the reaction was pre-incubated for 2 min at 42  $^\circ\text{C}$ . Finally,  $\text{MgCl}_2$  (3 mM),  $\text{MnCl}_2$  (1.5 mM; in  $\text{Mn}^{2+}$ -positive reactions), and SSII reverse transcriptase (10  $\text{U } \mu\text{L}^{-1}$ ) were added to the reaction mixture, and this was incubated for up to 8 h at 42  $^\circ\text{C}$ . For Bst reactions, PBS1 IR700 primer (5 pmol) was annealed to TNA template (10 pmol) in a final volume of 10  $\mu\text{L}$  in 1 $\times$  ThermoPol buffer by heating for 5 min at 95  $^\circ\text{C}$  and cooling for 10 min at 4  $^\circ\text{C}$ . dNTPs (500  $\mu\text{M}$ ) and  $\text{MgCl}_2$  (3 mM) were added to the primer template complex. Finally,  $\text{MnCl}_2$  (1.5 mM; in  $\text{Mn}^{2+}$ -positive reactions), and Bst DNA polymerase (1.6  $\text{U } \mu\text{L}^{-1}$ ) were added, and the reaction mixture was incubated for up to 8 h at 55  $^\circ\text{C}$ . Reaction products were analyzed by 10% denaturing PAGE and imaged by using a LI-COR Odyssey Clx. The amount of full-length product was quantified by using the LI-COR software package.

**Fidelity analysis:** DNA from reverse transcribed TNA was amplified by PCR (1 pmol) and ligated into a pJET vector following the manufacturer's protocol. The ligated product was transformed into chemically competent XL1-blue *Escherichia coli*, cloned, and sequenced (ASU Core Facility). Sequencing results were analyzed by using CLC Main Workbench. Sequences lacking the T to A watermark were discarded, as they were generated from the starting DNA template rather than replicated material. The error rate was determined by dividing the number of substitutions from the expected sequence over the total number of bases sequenced.

**Capillary electrophoresis SELEX:** For each round of selection, DNA template and FAM-labeled TNA were generated as described above. FAM-labeled TNA was suspended in 1 $\times$  binding buffer (20  $\mu\text{L}$ ; 10 mM Tris, 100 mM NaCl, 1 mM  $\text{MgCl}_2$ , pH 8.3) and folded by heating for 5 min at 95  $^\circ\text{C}$ , then cooling for 10 min at room temperature. CE was performed on a Beckman PA800 plus with an eCAP Neutral Capillary (50  $\mu\text{m}$  i.d., 60 cm long). The mobility of the TNA sample was determined in the absence of protein to determine the collection window. CE proceeded by first rinsing the capillary with NaOH (100 mM), then distilled water, followed by 1 $\times$  TKG buffer (25 mM Tris base, 177 mM glycine, 10 mM  $\text{KH}_2\text{PO}_4$ , pH 8.3, 0.2  $\mu\text{M}$  filtered) for 2 min each at 138 kPa (20 psi). TNA sample was injected for 4 s at 6.89 kPa (1 psi) and then separated for 20 min at 30 kV constant. Afterwards, thrombin (500 nM) was added to the TNA sample, and this was incubated for 30 min at 23  $^\circ\text{C}$ . CE-SELEX proceeded with five injections, collecting the bound protein complex from the unbound peak into 1 $\times$  ThermoPol buffer (50  $\mu\text{L}$ ). The collected TNA was then reverse transcribed for 2 h under optimized conditions (6 mM final  $\text{MgCl}_2$  and 1.6  $\text{U } \mu\text{L}^{-1}$  final Bst polymerase) in a 100  $\mu\text{L}$  final volume. After three rounds of selection, isolates were identified by cloning and sequencing the DNA as described above.

**RNA primer extension and reverse transcription:** For RNA primer extension, the protocol was identical to that in the TNA transcription section, with a substitution of Tgo-QGLK enzyme and NTP substrates for Kod-RI and tNTPs, respectively. Reverse transcription was performed with SSII following the manufacturer's recommended protocol.

**Dot blot solution binding analysis:** IR800-labeled TNA molecules were synthesized by primer extension and purified by denaturing PAGE, as described above. Aptamers were folded in binding buffer (10 mM Tris, 100 mM NaCl, 1 mM  $\text{MgCl}_2$ , pH 8.3) by denaturing for 5 min at 90  $^\circ\text{C}$  and then cooling for 30 min at room temperature. Folded structures were incubated with thrombin poised at concentrations spanning the expected  $K_D$  values (typically 1 nM–10  $\mu\text{M}$ ) at room temperature. Nitrocellulose, nylon, and filter paper were equilibrated in KOH (0.5 M, filtered) for 10 min, then Tris (0.5 M, pH 8) for 10 min, then 1 $\times$  binding buffer (10 mM Tris, 100 mM NaCl, 1 mM  $\text{MgCl}_2$ , pH 8.3) for 1 h. The dot blot apparatus was assembled with the equilibrated membranes and rinsed with 1 $\times$  binding buffer (3 $\times$ 100  $\mu\text{L}$ ). The protein-bound TNA molecules were partitioned away from the unbound fractions in a vacuum to pass the solution through the dot blot apparatus. After the solution passed, the wells were rinsed binding buffer (3 $\times$ 100  $\mu\text{L}$ ). After allowing all solution to pass through, the apparatus was disassembled, and both membranes were quantified by imaging on the LICOR. Dissociation constants were calculated with R by using non-linear least-squares regression to the equation  $F = F_{\min} + F_{\max} \times c / (c + K_D)$  where  $F$  is the fraction bound,  $F_{\min}$  and  $F_{\max}$  are the minimum and maximum bound fractions,  $K_D$  is the dissociation constant, and  $c$  is the protein concentration.



**Microscale thermophoresis binding analysis:** IR700-labeled TNA molecules were synthesized by primer extension and purified by denaturing PAGE as described above. Aptamers were folded in binding buffer (10 mM Tris, 100 mM NaCl, 1 mM MgCl<sub>2</sub>, pH 8.3) by denaturing for 5 min at 90 °C and then cooling for 30 min at room temperature. Folded structures at a final concentration of 2 nM were incubated with thrombin at concentrations spanning the expected  $K_D$  values (typically 1 nM–10  $\mu$ M) for 1 h at room temperature. Each titration sample was then loaded into a premium capillary (NanoTemper) and analyzed on a Monolith NT.115 Pico (NanoTemper; 20% MST power, 20% laser power). Binding isotherms were deduced by using NanoTemper's NT Analysis software.

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