Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

DNA display of folded RNA libraries enabling RNA-SELEX without reverse transcription

Iain S. MacPherson,*[a,b] J. Sebastian Temme[b] and Isaac J. Krauss*[b]

[a] *Department of Tropical Medicine, Medical Microbiology and Pharmacology, John A. Burns School of Medicine, University of Hawai`i at Manoa, 651 Ilalo St., Biosciences Building, Suite 325, Honolulu, Hawaii 96813-5525.*

[b] *Department of Chemistry, Brandeis University, 415 South St. MS 015, Waltham, MA 02454-9110, USA.*

Materials

Synthetic DNA oligonucleotides were purchased from Integrated DNA Technologies. Vent(exo-) DNA polymerase, Bst 2.0 warm start DNA polymerase, T7 RNA polymerase, T4 polynucleotide kinase, hydrophilic streptavidin magnetic beads, dNTPs, rNTPs, NheI-HF, EcoRI and BSA was purchased from New England Biolabs. DNA grade Sephadex G-50 was purchased from GE healthcare. Biotinylated D-Phe-Pro-Arg-chloromethylketone (PPACK) covalent inhibitor-bound human α-thrombin was purchased from Haematologic Technologies. GelRed DNA stain was purchased from Biotium, and Sybr Gold nucleic acid gel stain was purchased from Life Technologies. TOPO-TA cloning kit was purchased from Life Technologies. Chemicals, electrophoresis reagents and equipment were purchased from VWR, Sigma and Bio-Rad. γ-AT^{32}P was purchased from Perkin Elmer.

Table S1. Commercially purchased oligonucleotides.

Name	Sequence
Random library	5'CCGGGCTTTGTGTCACTTNNNNNNNNNNNNNNNNNNNNNNNNNNGCTCGTTCT CCTTCCCTCTCCTATAGTGAGTCGTATTACAGTTG
NheI library (NheI site underlined, selection oligonucleotide binding site highlighted)	5'CCGGGCTTTGTGTCACTTTACGTTCTTA**TGTTCTCACTCGCTA**GCTCGTTCT CCTTCCCTCTCCTATAGTGAGTCGTATTACAGTTG
Biotinylated T7 forward primer	5'Biotin-CAACTGTAATACGACTCACTATAGGAGA
IsodC reverse primer	5'isodC-CCGGGCTTTGTGTCACTT
Capture strand (rigidifier binding site underlined), capture sequence in bold	5'**GCTCGTTCTCCTTCCCTCTCC**TTTTTTTTTTCAACACCACAGACCAGTATAC CCAGAAATGACGCAAGCATAGACAAACGATTTAGACATGAGTGCCCCACACAAC GAACAAGCTTTTTTTTTTA-hexaethyleneglycol SPACER-CAACTGTAATACGACTCACTATAGGAGA
Capture strand rigidifier	5'GCTTGTTCGTTGTGTGGGGCACTCATGTCTAAATCGTTTGTCTATGCTTGCG TCATTTCTGGGTATACTGGTCTGTGGTGAA
NheI selection 15mer	5'Biotin-TGTTCTCACTCGCTA
Unmodified T7 forward primer	5'CAACTGTAATACGACTCACTATAGGAGA
Unmodified Reverse primer	5'CCGGGCTTTGTGTCACTT

Methods

Library construction

A starting random double stranded DNA (dsDNA) library was produced by standard PCR of a synthetic random single stranded DNA (ssDNA) library using a biotinylated forward primer and isodC reverse primer using vent-exo DNA polymerase (refer to Table 1 for sequence information). A 200 µl PCR reaction consisted of 85 pmol biotinylated forward primer, 80 pmol isodC reverse primer, 5 pmol negative strand template, 1X thermopol buffer, 4 units Vent(exo-) DNA polymerase, 200 uM each dNTP and cycling at 95 °C for 60 seconds followed by 6 cycles of 95 °C for 30 seconds, 57 °C for 30 seconds, and 72 °C for 10 seconds. Following PCR amplification, the reaction was quenched by addition of 5 µl of 0.5 M EDTA and 25 µl of 4 M NaCl. To remove the biotinylated strand, the crude PCR product was incubated with 0.28 mg (70 µl) hydrophilic streptavidin magnetic beads for 30 minutes. The beads were washed twice with wash buffer (20 mM Tris pH 8, 500 mM NaCl) and then resuspended in 40 µl 0.1 M NaOH for 4 minutes followed by application of a magnetic separator and transfer of the supernatant to 4 µl 1 M HCl and 1 µl Tris pH 8. For generation of the positive control isodC ssDNA, the same primers were used to amplify a starting NheI positive control template in a PCR reaction and the product was treated in an identical manner with streptavidin magnetic beads to generate the 5' isodC ssDNA library.

For incorporation of the capture arm into the random library, 23 µl of the isolated single stranded DNA (above) was combined with 40 pmol capture strand and 5 ul 10X thermopol buffer in a volume of 48 µl. For inclusion of the NheI positive control sequence at 1:1000, 2.3 µl of a 1/100 dilution of the NheI positive control sequence was also added. The mix was heated to 95 °C for 60 sec and cooled to 57 °C. The tube was placed on ice and 1 µl 10 mM dNTPs were added followed by the addition of 8 units Bst 2.0 warmstart DNA polymerase. The reaction was placed at 60 °C for 90 seconds and briefly cooled on ice before buffer exchange through a spin column loaded with DNA-grade Sephadex G50 equilibrated with 20 mM Tris pH 8, 4 mM $MgSO_4$.

To rigidify the capture arm with a complimentary 82mer oligonucleotide, 16 µl of the buffer-exchanged product was combined with 2 µl 10X RNA polymerase buffer and 1.3 µl rigidifying oligonucleotide (10 uM) and the mixture was incubated at 50 °C for 5 minutes. To remove unwanted biotinylated contaminants, the mixture was incubated with 0.08 mg hydrophilic streptavidin magnetic beads for 30 minutes and the supernatant retained. For transcription and RNA capture by its encoding DNA, the reaction was then cooled on ice and 0.2 µl DTT, 1 µl of 10 mM rNTPs and 5 units T7 RNA polymerase was added and the reaction was incubated at 37 °C for 8 minutes. In the NheI-based selection experiment, a negative control experiment was also performed in which the transcription step was omitted. The negative control sample was otherwise treated identically to the transcribed library.

Selection experiments

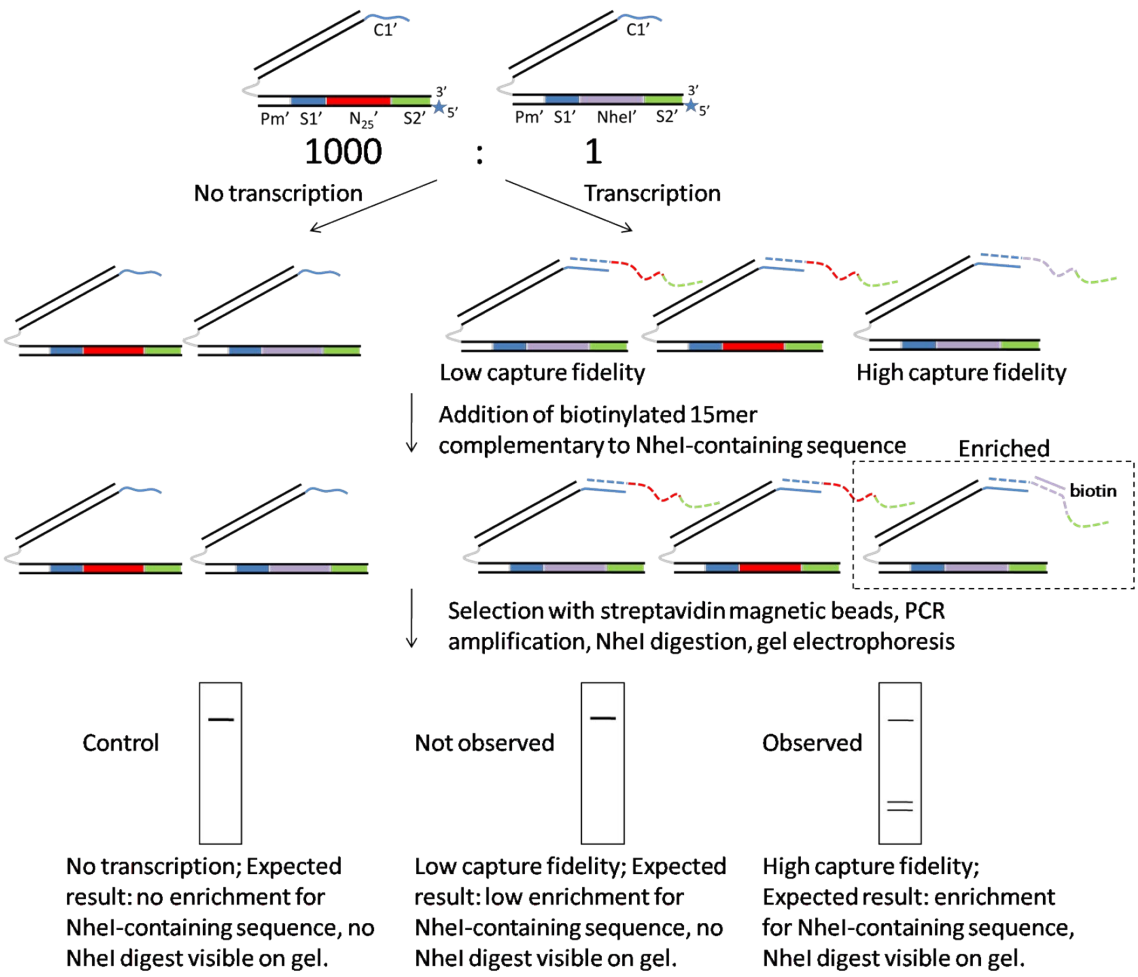
Control experiment with spiked library: The spiked NheI-containing sequence was selected by binding to a short complementary sequence. Briefly, the transcription reaction (or non-transcribed reaction in the negative control) was diluted into 50 µl with water and incubated with 50 nM of the biotinylated complementary 15mer for 10 minutes at 37 °C and then mixed with 0.1 mg hydrophilic streptavidin magnetic beads for 20 minutes. The supernatant was removed and the beads were resuspended in 30 µl elution buffer (20 mM Tris pH 8, 50 mM

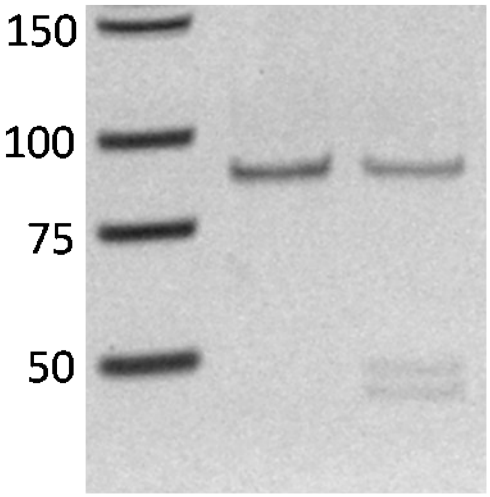
NaCl, 10% Tween-20, 150 ug/ml BSA) and placed in a 70 °C dry bath for 5 minutes, after which the supernatant was used in a PCR amplification reaction.

Thrombin selection: The RNA-displayed DNA library was selected for binding to biotinylated PPACK-bound human α-thrombin by incubation at varying thrombin concentrations and times, starting with 10 nM for 1 hour in round 1 and decreasing to 1 nM for 5 minutes in round 10 (see table S2). Thrombin-bound library was recovered by incubation with 0.1 mg streptavidin magnetic beads for 30 minutes, followed by washing with 100 µl and 150 µl selection buffer (20 mM Tris pH 8, 150 mM NaCl, 2 mM MgSO4, 0.05% Tween-20). The beads were resuspended in 30 µl elution buffer and placed in a 95 degree dry bath for 3 minutes before removal with a magnetic rack, after which the supernatant was used in a PCR amplification reaction.

Amplification: For either type of selection step, the 30 µl eluted binders (above) were added to a 230 µl PCR reaction mix containing 35 pmol biotinylated forward primer, 35 pmol isodC reverse primer, 1X thermopol buffer, and 200 uM dNTPs. 30 µl of the 230 µl pre-mix was added to a single PCR tube, after which 0.6 units Vent(exo-) polymerase was added. The reaction was mixed, 10 µl amounts were distributed to two other tubes and all three tubes were amplified at the above described thermal cycling conditions (95 °C for 30 seconds, 57 °C for 30 seconds, 72 °C for 10 seconds) for varying cycle numbers, typically 10, 12 and 14 cycles. 5 µl from each tube was run on a 2% agarose DNA gel and the optimal cycle number was determined based on the appearance of 87 bp product. Then, 4 units of vent(exo-) polymerase was added to the remaining 200 µl of pre-mix and the reaction distributed over 4 tubes and amplified with the optimized cycle number, yielding double stranded DNA to be used in the next round of library generation and selection. Alternatively, for the NheI susceptibility studies, 4 µl of the PCR product was added directly to a restriction digest containing 1X Cutsmart buffer and 10 units of NheI HF in a total volume of 20 µl. 5 µl of the digestion product was run on a 6% non-denaturing polyacrylamide gel and stained with GelRed DNA stain.

Figure S1. Faithful capture of RNA by its encoding DNA demonstrated by a simple hybridization-based selection.





Lane 1, ladder; lane 2, NheI-digested PCR amplicon from non-transcribed library (Control); lane 3, NheI-digested PCR amplicon from transcribed library. Amplifications in lanes 2 and 3 were performed on recovered library after the second round of selection.

Table S2. Thrombin Aptamer Selection Conditions

Round	[Thrombin]	binding time	# PCR cycles for recovery
1	10 nM	1 hour	14
2	10 nM	1 hour	14
3	10 nM	1 hour	14
4	10 nM	1 hour	14
5	10 nM	1 hour	14
6	10 nM	1 hour	14
7	10 nM	1 hour	12
8	10 nM	30 min	10
9	10 nM	5 min	11
10	1 nM	5 min	14

Cloning and sequencing

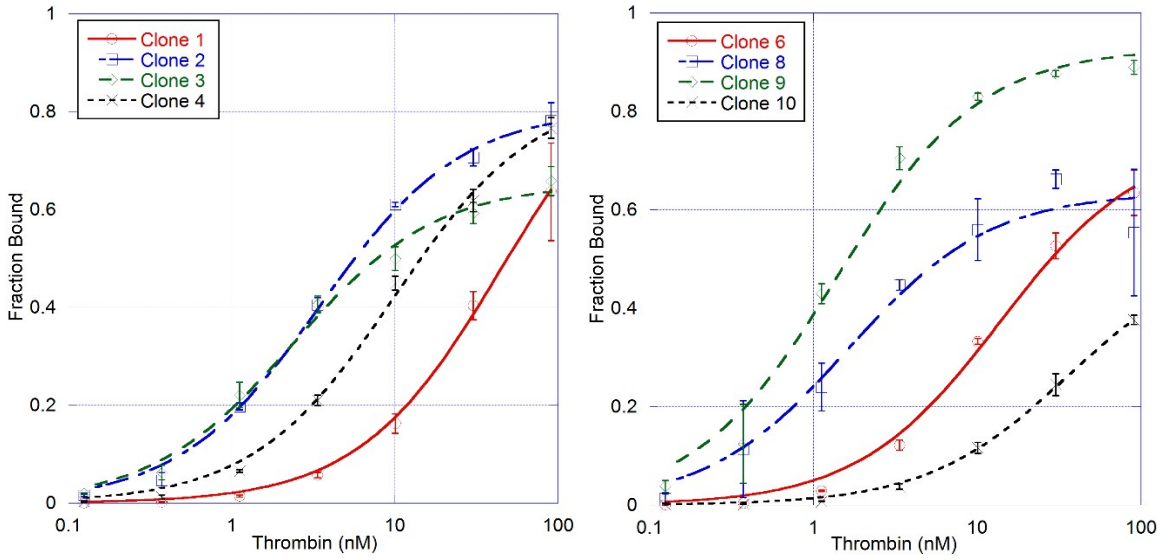
The thrombin binding library recovered after round 10 was amplified using unmodified forward and reverse primers and the PCR product was cloned into plasmid pCR2.1 using a TOPO-TA kit. Ten E. coli colonies were grown overnight in liquid culture and the plasmids isolated using a Qiagen plasmid mini-prep kit and submitted for sequencing.

Table S3. Full sequences and binding constants of oligonucleotides tested in filter binding assay

Clone ID	Sequence	K_d (nM)	Fb_{max}
1	GGAGAGAGAAGGAGAACGAGCUGUUACUCACAAAAUAGCGAAGACUAAGUGACACAAAGCCCGG	44.7 ± 4.6	95.6 ± 4.4
2	GGAGAGGUAAGUAGAACGAGCCCGGCGUCACAAGAUAGACAAAACUAAGUGACACAAAGCCCGG	3.5 ± 0.3	80.6 ± 1.6
3	GGAGAGGGAAGGAGAACGAACCGGGACUCACAAGAUAGACAAUACUAAGUGACACAAAGCCCGG	2.4 ± 0.3	65.3 ± 1.9
4	GGAGAGGGAAGGAGAACGGCUGCGGAUAACAAGAUAGCAAAGACUAAGUGACACAAAGCCCGG	10.0 ± 0.9	84.5 ± 2.3
5	GGAGAGGUAGGAGAACGAGCCUACCGUAAGAGAACGAGCAGACUCAAGUGACACAAAGCCCGG	NB	ND
6	GGAGAGGGAAGGAGAACGAGCCGGGACACUAAGGAACAUAAAAGUUAAGUGACACAAAGCCCGG	13.8 ± 1.9	74.5 ± 3.2
7	GGAGAGGGAAUGAUAACGAGCCCGAAGCUCGGAGAAGCACAGAAGCAAGUGACACAAAGCCCGG	NB	ND
8	GGAGAGCGAAGAGAACGAGCGGGAUUGCACAAGAUAGCGUAGACUAAGUGACACAAAGCCCGG	1.6 ± 0.4	63.5 ± 3.3
9	GGAGAGGGAAAGAGAACGAGCGGGUGUUCACAAGAUAGAGUAGACUAAGUGACACAAAGCCCGG	1.4 ± 0.2	92.3 ± 3.1
10	GGAGACGGAAGGAUAACGAGCGCUUUGACACAAGAUACAAUAUAGUAAGUGACACCAAGCCCGG	36.4 ± 2.8	52.9 ± 1.7

Black = sequences derived from random sequence region. Green = complement of capture sequence. Blue = complement of reverse primer. NB = no binding; ND = not determined. Sequences in green contain slight mutations from the capture sequence complement present in the starting library, possibly accumulated during selection. K_d and Fb_{max} values are derived in Kaleidagraph by fitting the equation Fb = (Fbmax)*[thrombin]/(K_d + [thrombin]) to the plot of average triplicate Fb (fraction bound) data vs. thrombin concentration. Errors in the table are standard errors of the fitted parameters reported by Kaleidagraph's nonlinear curve fit function.

Figure S2. Filter binding curves



RNA preparation and filter binding assay

Plasmids isolated above were digested with EcoRI restriction enzyme and used as template in a PCR reaction with T7 forward primer and isodC reverse primer, resulting in dsDNA template suitable for transcription. RNA from clones 1-10 was generated by transcription of the PCR products in 50 µl reactions, each containing 1X transcription buffer, 5 µl crude PCR product, 500 uM NTPs, and 15 units T7 RNA polymerase, incubated for 4 hours at 37 °C. The RNA products were run on a 12% polyacrylamide gel and the 64-base band excised. The gel slices were crushed and soaked in 100 µl water at room temperature overnight, and the supernatant was passed through a desalting column loaded with sephadex G-50 as a final cleanup step.

Binding buffer was prepared as 20mM Tris pH 7.5, 150 mM NaCl, 2 mM $MgSO_4$, and 50ug/mL BSA. RNA products were 5'-radiophosphoylated using T4 polynucleotide kinase, 200 nM γ-AT^{32}P in 30 µl of 1X PNK buffer. After 30 minute at 37 °C, the reactions were passed through a desalting column loaded with sephadex G-50 to remove salts and excess γ-AT^{32}P. 10-100 fmol of the 5'-^{32}P labeled RNA was diluted into 50 µL of binding buffer and heated to 70 ^{o}C for 3 minutes, then cooled to RT to promote proper folding. Thrombin was serially diluted in binding buffer from 50 µM stock solution. For each clone studied, 50 µL aliquots of each thrombin dilution were pipetted into low retention 0.5 mL tubes (USA Scientific). 5µl of the labeled and folded RNA was then added to each dilution and incubated at 22^{o}C for 30 mintutes. Following incubation, the aliquots were passed through a 96 well sandwich filtration apparatus loaded with activated nitrocellulose/PVDF. Each well was then washed with 200ul of freshly prepared binding buffer. The membranes were then dried under vacuum, wrapped in cellophane, and exposed to a phosphorimaging plate. The plate was imaged after an appropriate length of time for adequate exposure. The data (Figure S2) were then fit to $F_{bound}=(F_{max}[Thrombin])/(K_d+[Thrombin])$. The results are tabulated in Table S3.

Truncation of Clone 9 and Thrombin Affinity Measurements by BLI and Filter Binding

We examined the binding of immobilized truncated clone 9 to thrombin in real time via biolayer interferometry (BLI) using a ForteBio BLItz instrument. Truncated RNA Clone 9 (See Figure S4), modified with a 3'-15-atom triethylene glycol (TEG) spacer arm and biotin tag, was synthesized by IDT and immobilized on a streptavidin sensors. Association and dissociation of thrombin was observed at several concentrations (0.5, 1, 2, 4, 8, 16, and 32 nM). A new biosensor was used for each curve and reproducibility between sensors was achieved by integrating a preconditioning step prior to aptamer loading. Binding experiments were performed as follows: Each biosensor was hydrated in buffer 1 (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM $MgSO_4$, 0.20 mg/ mL BSA, 0.02% Tween-20) for 20 minutes. Shaker speed was maintained at 2600 rpm during the experiment. After loading the biosensor onto the instrument, a 30s baseline in buffer 1 was followed by a 240s preconditioning step in Buffer 2 (50 mM NaOH, 1M NaOH). Equilibration of the biosensor in buffer 1 was achieved by two sequential 30s baselines in buffer 1. The aptamer was loaded during a 90s step in load buffer (Buffer 1 with 25 nM folded aptamer) yielding a response of 0.4nm. Preassociation equilibration of the biosensor was achieved by two sequential 30s baselines in buffer 3 (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM $MgSO_4$, 2.0 mg/mL BSA, 0.1% v/v Tween-20). Association of thrombin in buffer 3 was monitored for 360s and dissociation was monitored in blank buffer 3 for 300s. All data was referenced against an aptamer-loaded sensor exposed to blank buffer to subtract the effect of buffer changes. Data were exported into Graphpad Prism and curve fit to a 1:1 binding model yielding rate constants of $k_{on} = (91.9 \pm 0.1) \times 10^4\ M^{-1}\ s^{-1}$ and $k_{off} = (4.12 \pm 0.02) \times 10^{-4}\ s^{-1}$, corresponding to a K_D of 448 ± 2 pM. R_{max} was globally fit, yielding a value of 1.477 ± .001. Errors reported are the standard errors of the curve fit.

Equations Used in GPP:

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Radioligand=HotNM*1e-9
Kob=[Radioligand]*Kon+Koff
Kd=Koff/Kon
Eq=Rmax*radioligand/(radioligand + Kd)
Association=Eq*(1-exp(-1*Kob*X))
YatTime0 = Eq*(1-exp(-1*Kob*Time0))
Dissociation= YatTime0*exp(-1*Koff*(X-Time0))
Y=IF(X<Time0, Association, Dissociation) + NS
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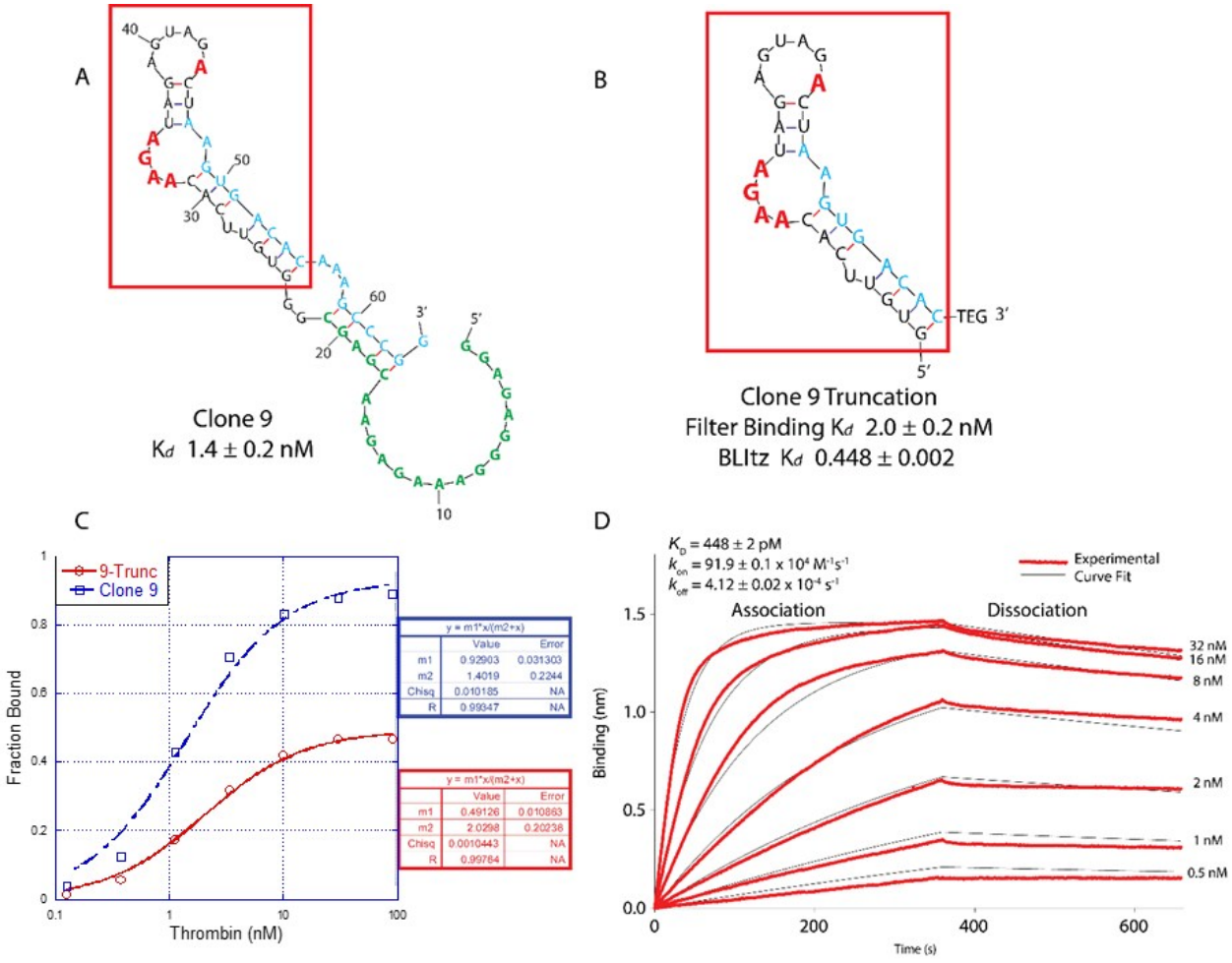
Figure S4. Truncated Clone 9 Thrombin Affinity Measurements by BLI and Filter Binding

A) Mfold predicted secondary structure of Clone 9 and B) of truncated Clone 9. C) Filter binding data of Clone 9 and the truncated form used in BLI (Biolayer Interferometry). D) BLI experimental and curve fit data.



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