Electronic supplementary information

Selection of a self-cleaving ribozyme activated in chemically and thermally denaturing environment

Lingzi Ma, Zhicheng Huang, and Juewen Liu*

Department of Chemistry, Waterloo Institute for Nanotechnology, University of Waterloo,

Waterloo, Ontario, N2L 3G1, Canada

Email: liujw@uwaterloo.ca

Materials and Methods

Oligonucleotides and chemicals. All the oligonucleotide sequences involved in the selection experiment were purchased from Eurofins Genomics LLC (Table S1). Chemical reagents including T7 RNA polymerase, 10X RNAPol reaction buffer, NTPs mixture, ProtoScript® II reverse transcriptase, 5X ProtoScript II buffer, dNTPs mixture, 0.1 M DTT, low range ssRNA ladder, 2X RNA loading dye, and *Taq* DNA polymerase with standard *Tag* buffer were purchased from New England BioLabs. SYBR gold dye (10,000×) was purchased from Thermo Fisher Scientific. The water used in *in vitro* selection experiments was pre-treated with RNA*secure*™ reagent (Thermo Fisher Scientific).

***In vitro* selection.** The initial RNA library was *in vitro* transcribed from a double-stranded DNA template using T7 RNA polymerase. To generate the dsDNA template, one cycle of PCR extension (95℃ for 2 min, 95℃ for 20 s, 59℃ for 20 s, and 72℃ for 30 s) was performed using single-stranded antisense DNA template (200 pmol) and primer 1 (200 pmol). The dsDNA template contained 103 base pairs as confirmed on a 2% agarose gel (Figure S6A). The extended DNA template was then purified with a PCR clean-up kit (IBI Scientific) and resuspended in water. The *in vitro* transcription was conducted in a 20 μL reaction (~100 pmol dsDNA template, 2 mM NTPs, 5 U/μL T7 RNA polymerase) by incubating at 37℃ for 2 h. The transcription product was precipitated by incubating with 5 M ammonium acetate (1× volume) and ethanol (2.5× volumes) at -20 ℃ for 1 h. After centrifugation (at 4℃, 14000 rpm, >30 min), the supernatant was carefully removed followed by twice washing with 70% ethanol (ice cold). The air-dried pellet was resuspended and purified by 10% denaturing polyacrylamide gel electrophoresis (dPAGE, 400V, 2 h). The gel was stained in 1× SYBR gold dye (ThermoFisher) and then imaged under a Bio-Rad Chemi-Doc MP imaging system. The intact RNA precursors (80-nt) were extracted from the gel and ethanol precipitated. The RNA precipitant was stored at -20 ℃ until further use.

In each selection, the RNA library was resuspended in 20 μL of selection buffer (50 mM MES, pH 6, 100 mM Na^+, 2 mM EDTA•2Na^+) at room temperature for 2 h. After incubation, the reaction was quenched with 1× RNA loading dye (NEB) at 65℃ for 10 min. The RNA loading dye (1×) contains 47.5% formamide, 0.01% SDS, 0.01% bromophenol blue, 0.005% xylene cyanol, and 0.5 mM EDTA. The RNA cleavage products (~67 to ~73-nt) were carefully isolated by aligning with ssRNA ladder on 10% dPAGE. The cleavage products were extracted by crush/soaking the gel slices and were ethanol precipitated. Next, the cleaved RNAs (10 μL) were

annealed with primer 3 (100 pmol) by incubating at 65℃ for 5 min. After chilling on ice, 0.5 mM dNTPs, 10 mM DTT, and 10 U/μL ProtoScript® II reverse transcriptase were added resulting in a total of 20 μL. The cDNA synthesis reaction was performed under 42℃ for 1 h followed by inactivation at 80℃ for 5 min. The primer 3 restores the lost 3'-fragments after the cleavage reaction. The reverse transcribed product was directly monitored using real-time PCR. As optimized in Figure S6B, the volume of cDNA product was kept ≤1/20 of the PCR reaction volume to eliminate the interference of residues from reverse transcription. In each round, the fractional cycles ($C_{1/2}$) at which reaction fluorescence reaches half of maximal (F_{max}) was used for PCR amplification (95℃ for 2 min, 95℃ for 20 s, 59℃ for 20 s, and 72℃ for 30 s). The optimized cycle# and template length were confirmed with 2% agarose gel (as shown in Figure S6C). Two rounds of PCR (primer 1 and 2) were performed to produce sufficient dsDNA template which is purified by isopropanol precipitation. After resuspended in water, ~200 pmol dsDNA was used for the next round of selection.

Sequencing sample preparation. To prepare the sample for deep sequencing, PCR1 was performed to generate the full-length library. Next, PCR2 was performed to introduce specific index sequences into the library for the Illumina sequencing technology. Instead of primer 1 and 2, the forward primer (S505) and the reverse primer (N702) were used with their sequences listed in Table S1. The PCR product was then purified with 2% agarose gel (120 V, 50 min). A gel/PCR DNA fragment extraction kit (IBI Scientific) was used to extract the DNA library from the gel. Finally, the purified DNA sample was eluted in 20 μL of Milli-Q water. The DNA concentration measured with a NanoDrop spectrophotometer was ~38.1 ng/μL. The sample was shipped to McMaster University for deep sequencing.

Characterization of the cleavage activity. After sequence analysis, single-stranded DNA templates (Table S1) encoded promising sequences were extended by PCR and *in vitro* transcribed into RNA sequences. The 80-nt RNA products were further purified by 10% dPAGE and ethanol precipitated. The purified ribozyme can be resuspended in water for activity assays. For cleavage reactions performed in 50% formamide, 5 μL pure formamide (≥ 99%) and 50 mM Tris•HCl buffer (pH 7.5) was added to a total of 10 μL reaction volume. Except for the metal dependency assays, a final concentration of 1 mM EDTA•$2Na^+$ was added in all reactions. After incubation, the self-cleavage reaction of Rn2 was quenched by cooling at -20°C. The reaction products were then visualized and analyzed on 10% dPAGE. Kinetic data were fit with the first-order equation,

$\%P_{cleavage,t} = \%P_{max}(1 - e^{-K_{obs}t})$, where $\%P_{max}$ is the maximum cleavage yield at the end of the reaction and K_{obs} is the cleavage rate constant.

Table S1. The oligonucleotide sequences used in the *in vitro* selection experiment.

Antisense DNA template (103-nt)	5′GGCCATCTTCCTATAGGCTTGTGCCTAN$_7$AGTCAACACTN$_{20}$GATGGCCTCTCTTCCC TATAGTGAGTCGTATTAGAATTC
Sense DNA template (103-nt)	5′GAATTCTAATACGACTCACTATAGGGAAGAGAGGCCATCN$_{20}$AGTGTTGACTN$_7$TAGG CACAAGCCTATAGGAAGATGGCC
Primer 1 (39 nt)	5′GAATTCTAATACGACTCACTATAGGGAAGAGAGGCCATC
Primer 2 (21 nt)	5′GGCCATCTTCCTATAGGCTTG
Primer 3 (27 nt)	5′GGCCATCTTCCTATAGGCTTGTGCCTA
S505 primer	5'AATGATACGGCGACCACCGAGATCTACAC-GTAAGGAG- ACACTCTTTCCCTACACGACGCTCTTCCGATCT-NNNN-GGGAAGAGAGGCCATC
N702 primer	5'CAAGCAGAAGACGGCATACGAGAT-CTAGTACG- GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-GGCCATCTTCCTATAGGCTTG
Rn2 DNA template	5′GGGAAGAGAGGCCATCAGGTCACTCTCCCGCCCCCCAGTGTTGACTACCCCTTTAGG CACAAGCCTATAGGAAGATGGCC
Rn2.1 DNA template	5′GGGAAGAGAGGCCATCAACGGTCTCCGGTTCCCACTAGTGTTGACTACCCCATTAGG CACAAGCCTATAGGAAGATGGCC
Rn2.2 DNA template	5′GGGAAGAGAGGCCATCAGGTCGACTCGTCCCCGCCCAGTGTTGACTACCCAAATAGG CACAAGCCTATAGGAAGATGGCC

Note: Red coloured domain represents the T7 promotor sequence; Underlined domain is to ensure T7 promotor in duplex; Grey coloured domain is for primer binding.

Table S2. Top 8 families (from 5′ to 3′) after sequence alignment present in the final library. The red and blue regions represent the N_{20} and N_7 regions, respectively.

2.GGGAAGAGAGGCCATCAGGTCACTCTCCCGCCCCCCAGTGTTGACTACCCCTTTAGGCACAAGCCTATAGGAAGATGGCC(98 reads)

5.GGGAAGAGAGGCCATCCCTCGGTCTCCTAGCTTCCCAGTGTTGACTACTCCCCTAGGCACAAGCCTATAGGAAGATGGCC(69 reads)

14.GGGAAGAGAGGCCATCCTATCATGGTCTCCGCTCCCAGTGTTGACTCCACACGTAGGCACAAGCCTATAGGAAGATGGCC(55 reads)

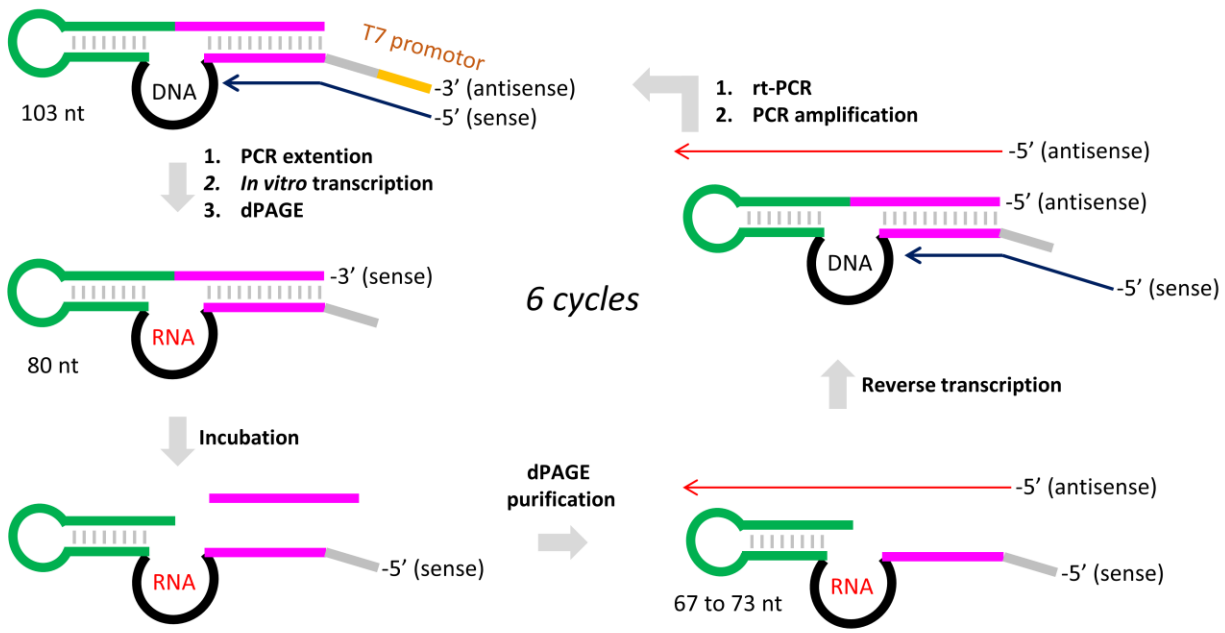
17.GGGAAGAGAGGCCATCATGGTCTCCGATCCCATCCCAGTGTTGACTCCTGACATAGGCACAAGCCTATAGGAAGATGGCC(52 reads)

19.GGGAAGAGAGGCCATCCCGGTCTCACGTTTCCCAGAGTGTTGACTCTCCCCTTAGGCACAAGCCTATAGGAAGATGGCC(50 reads)

20.GGGAAGAGAGGCCATCCATCTGGTCTAACTTCCCGAAGTGTTGACTCCCCCTCTAGGCACAAGCCTATAGGAAGATGGCC(50 reads)

23.GGGAAGAGAGGCCATCCCGGTCTCACGTTTCCCAGAGTGTTGACTCTCCCCTTAGGCACAAGCCTATAGGAAGATGGCC(48 reads)

24.GGGAAGAGAGGCCATCCGCGCACTCATTCCCAACCCAGTGTTGACTCATCCTTAGGCACAAGCCTATAGGAAGATGGCC(48 reads)



Scheme S1. The *in vitro* selection procedure for self-cleaving ribozymes. In each selection round, the RNA library (80-nt) was generated by *in vitro* transcription followed by dPAGE purification. The purified RNAs were incubated in the selection buffer (50 mM MES, pH 6, 100 mM NaCl, 2 mM EDTA•2Na^{+}) at room temperature for 2 h. Afterwards, the downstream products with lengths between ~67 to 73-nt were purified by dPAGE and amplified by reverse transcription PCR.

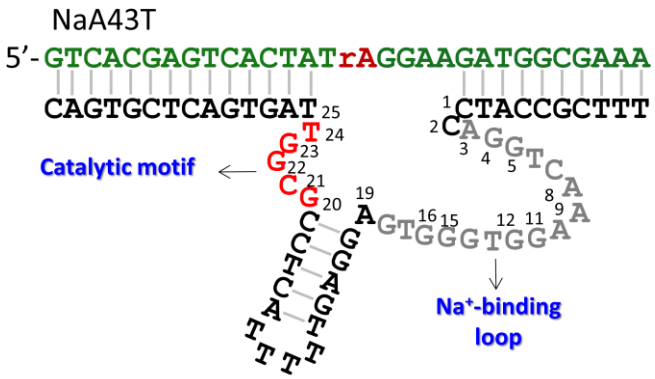


Figure S1. The secondary structure of the NaA43T DNAzyme.[1, 2]

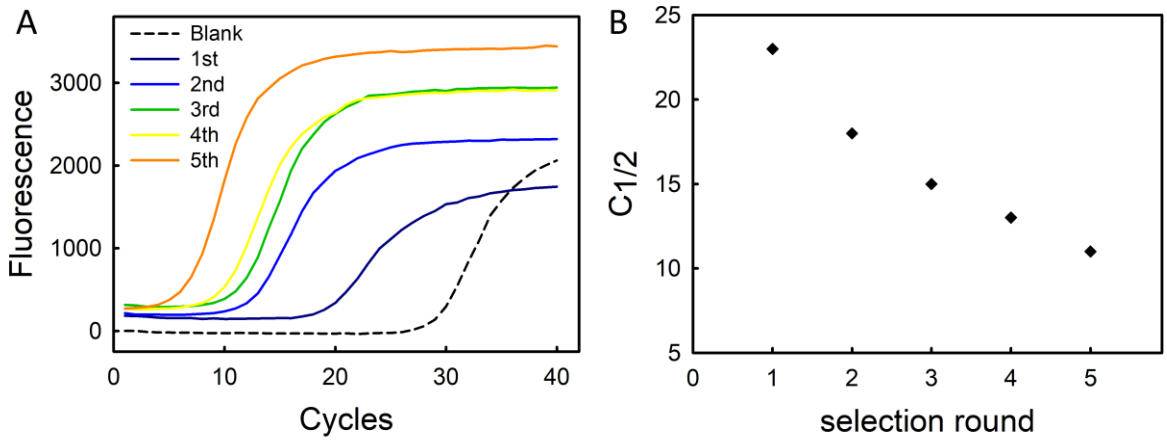


Figure S2. (A) Real-time PCR used to monitor the gradual increase in RNA population in the library along with the selection progress. (B) The fractional cycles ($C_{1/2}$) based on the real-time PCR, at which the reaction fluorescence reached half of maximal (F_{max}).

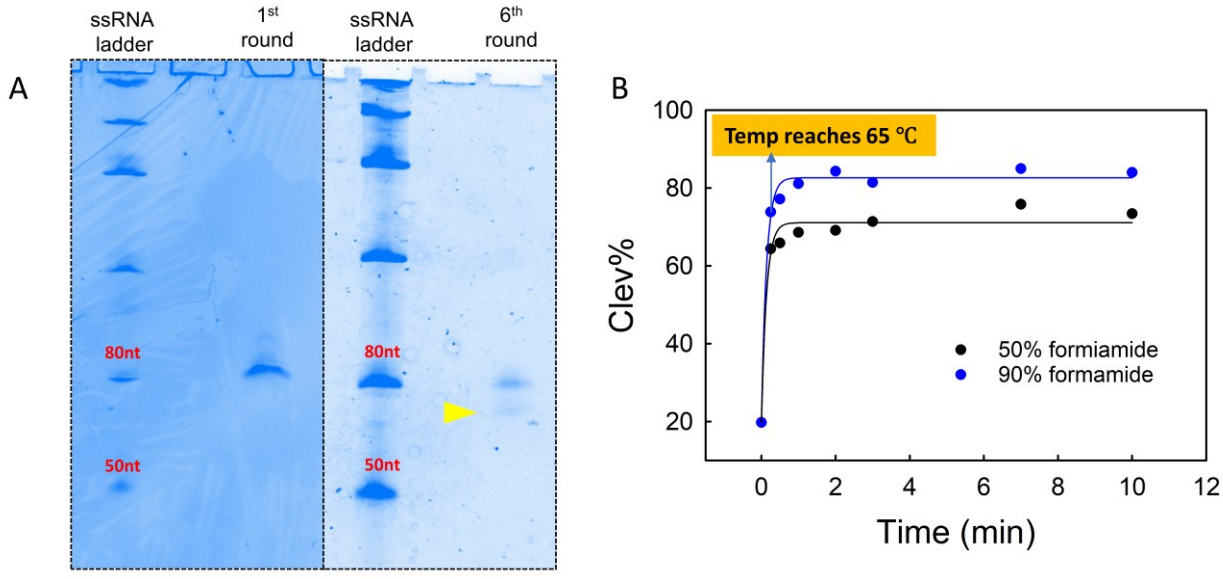


Figure S3. (A) Gel images showing the cleavage products of the first and sixth round after the selection step (the product band indicated by the yellow arrowhead). (B) The cleavage% measured at different time points in the presence of 50% and 90% formamide at 65°C.

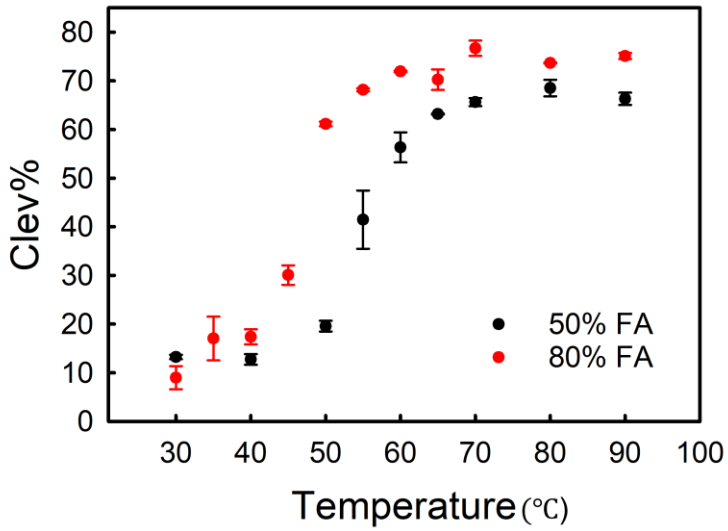


Figure S4. The cleavage% of Rn2 measured in 50% or 80% formamide during a slow heating process. The reaction was gradually heated from 30°C to 90°C. The reaction was remained for 20 s at each temperature point. An accumulated cleavage% was measured after being quenched at each temperature point.

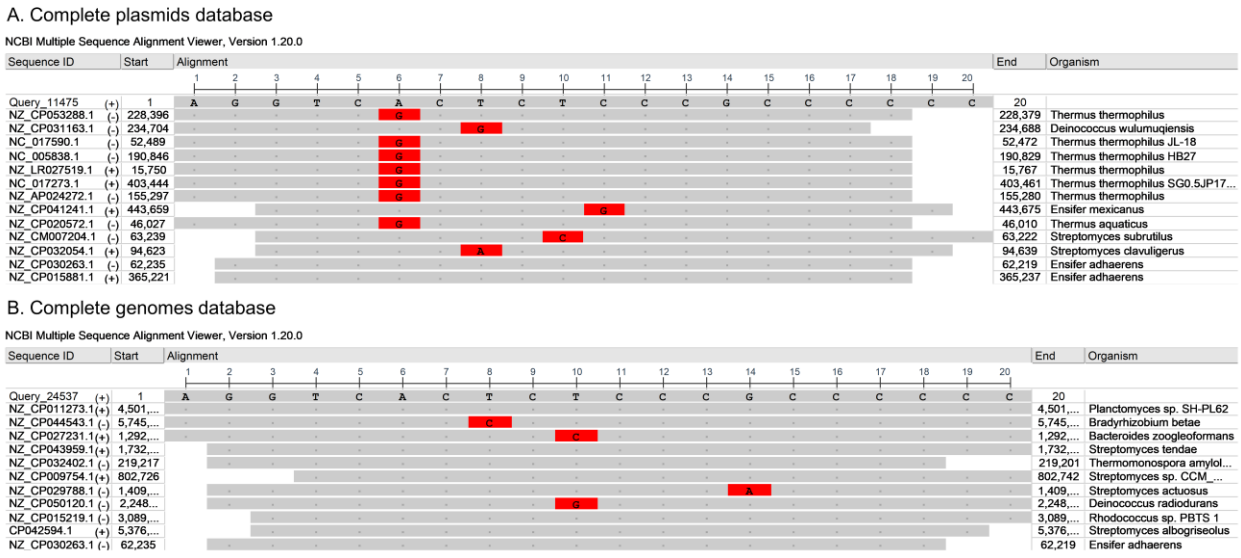


Figure S5. Nucleotide NCBI-BLAST results showing the alignments of Rn2 motif in complete plasmids (A) or genomes (B) databases of microbes. The N_{20} region of Rn2 ribozyme was used as the input sequence.[3]

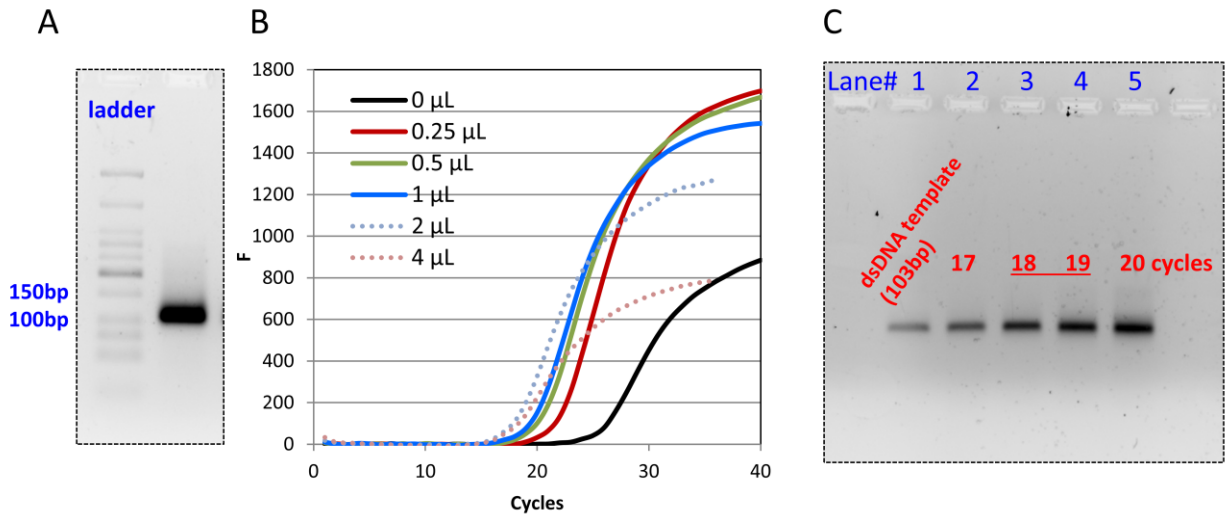
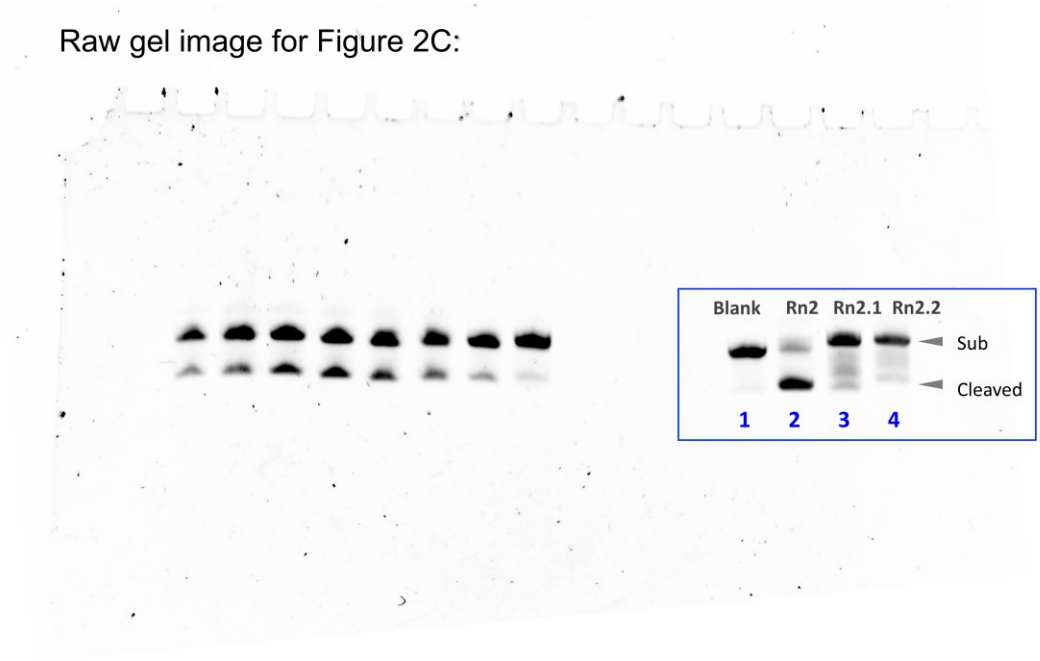
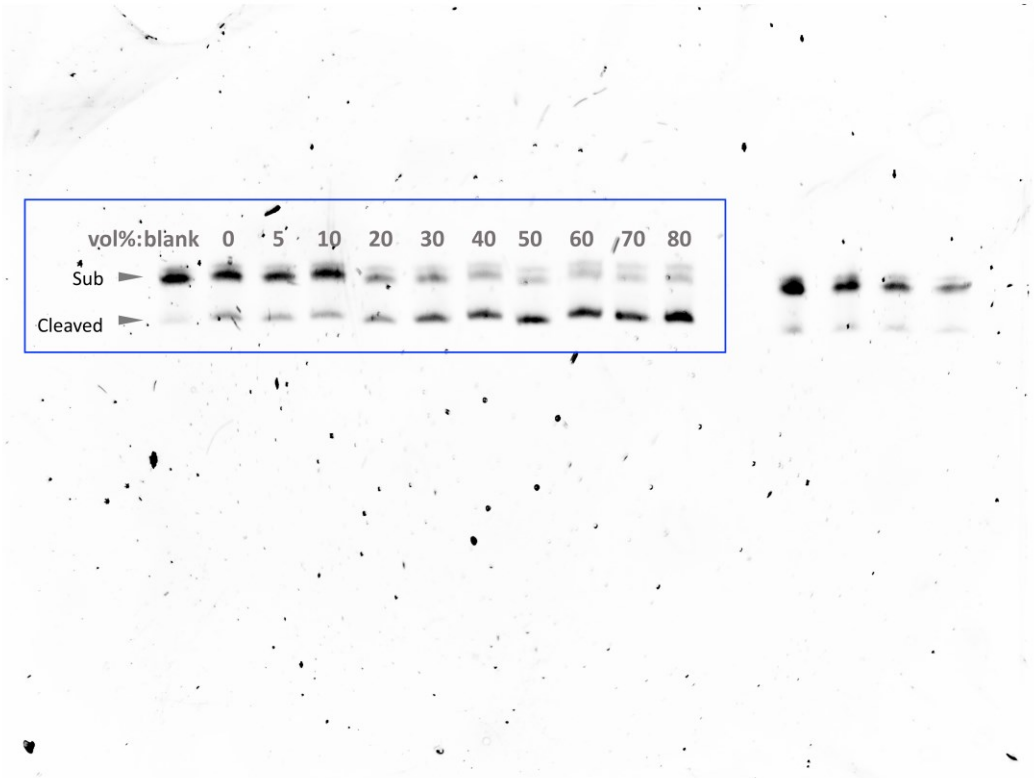


Figure S6. (A) A gel micrograph showing the extended dsDNA template use for *in vitro* transcription with a length of 103 bps. (B) Real-time PCR for optimizing the cDNA volume used in PCR reaction. The volume of the cDNA was kept $\leq$1/20 of the PCR reaction volume to eliminate the interference of residues from the reverse transcription reaction. (C) The optimized PCR cycle number and template length were confirmed with 2% agarose gel for each round of selection.

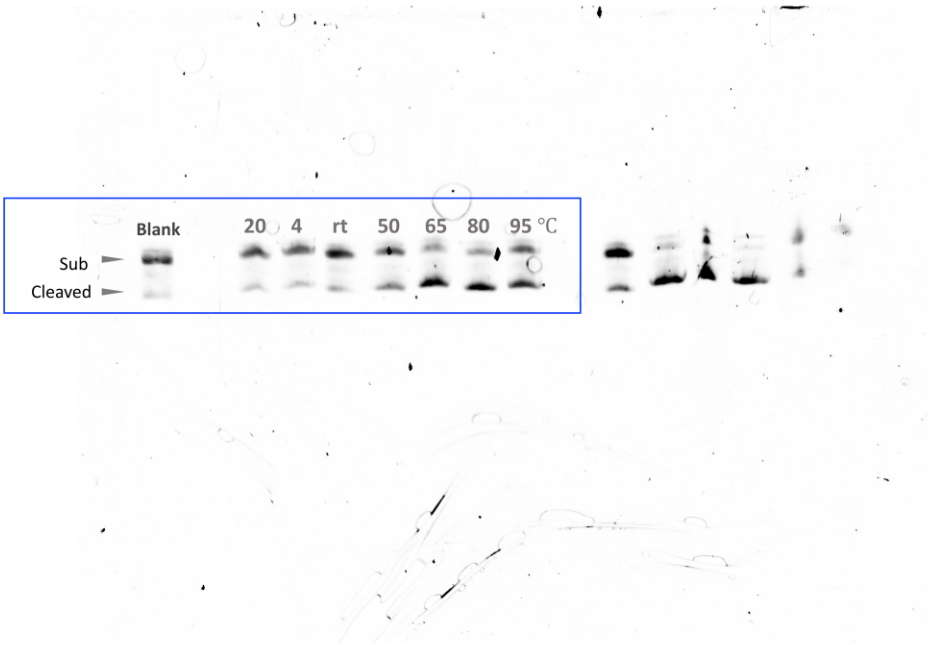
Raw gel image for Figure 2C:



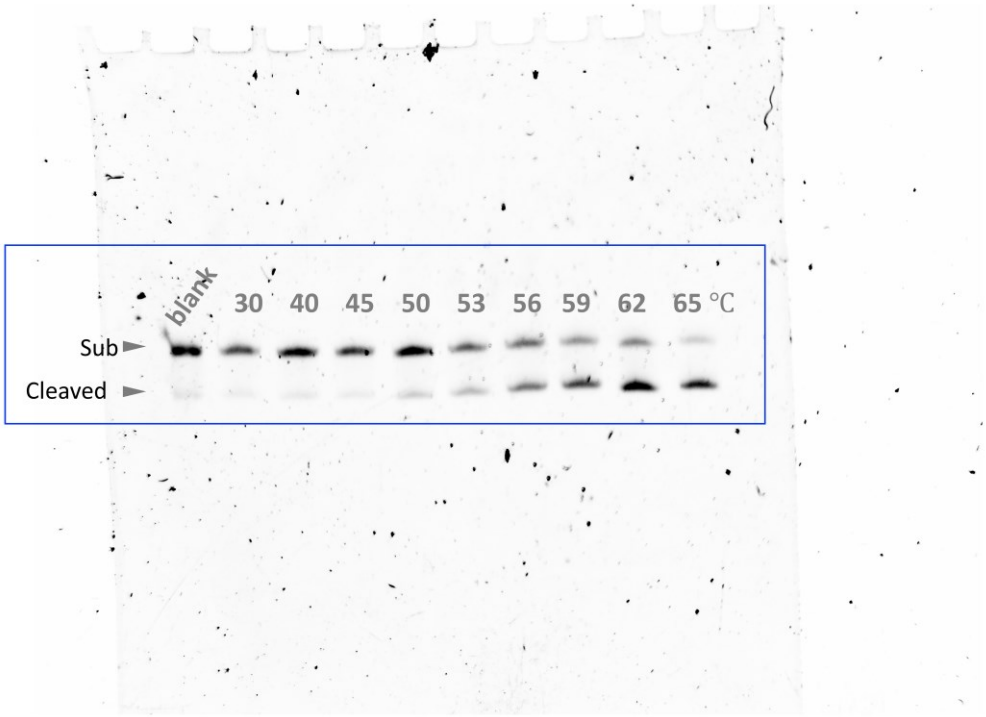
Raw gel image for Figure 3A:



Raw gel image for Figure 3B:



Raw gel image for Figure 3C:



References

1. S.-F. Torabi, P. Wu, C. E. McGhee, L. Chen, K. Hwang, N. Zheng, J. Cheng and Y. Lu, *Proc. Natl. Acad. Sci. U.S.A.*, 2015, **112**, 5903-5908.
2. W. Zhou, Y. Zhang, P.-J. J. Huang, J. Ding and J. Liu, *Nucleic Acids Res.*, 2016, **44**, 354-363.
3. S. F. Altschul, T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller and D. J. Lipman, *Nucleic Acids Res.*, 1997, **25**, 3389-3402.