Supplementary material for

Toehold mediated strand displacement to measure released product from self-cleaving ribozymes

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Algorithm to design ribozymes inducible by an input

An EA is a search and optimization method that simulates evolution within a computational environment. It operates on a population of candidate solutions (individuals) to a problem. Generally, an EA terminates when one or more satisfactory solutions to the problem are found, stagnation occurs or, an upper limit to computational resources is reached.

The EA is composed of six procedures: 1. Configuration, 2. Initialization, 3. Fitness evaluation, 4. Parent selection, 5. Mutation and 6. Survivor selection.

Configuration is a five-step process. First, the user provides the m inputs to be cleaved or not by the RNA-regulated cis-acting HHR. User input is used to specialize a *generic* HHR 'structural template' into a *specific* structural template (ST). Second, the ST with added constraints is used to generate a 'segment dependency graph' (or SDG). Third, the SDG is converted to a 'base dependency graph' (BDG): each segment-pair of n nucleotides is converted to n-pairs of nucleotides, and each unpaired segment of n nucleotides is converted to n unpaired nucleotides. This is followed by folding constraints generation. Next is **initialization**. Each individual is represented by one 'gate strand' (i.e., ribozyme sequence) and input strands. The strands are filled with bases respecting the constrained BDG. Then comes fitness evaluation.

Fitness evaluation: First, gate folding is carried out. Folding folds the gate strand n times, each time asserting one of the n different constraint strings. The result is n base-pairing probability matrices, each with its minimum free energy (MFE) secondary structure. Second, for each of the n states, n logic scores are computed. Each score reflects how close the folding of the gate strand is to its ideal folding. These (n) logic scores are used to compute the ON and OFF logic scores for the ribozyme, which are then used to compute the ribozyme's overall objective score. All three scores comprise a score tuple. Since each individual has a score tuple, a multi-objective ranking scheme is used to assign every individual a rank. Finally, totally inviable HHR designs are summarily deleted.

Fitness evaluation is followed by **Termination** if either the computational resources (essentially, time) are exhausted, or a sufficiently large set of diverse solutions are found. If the EA does not terminate, it proceeds to **Parent Selection** where each parent produces one offspring, via mutation. **Mutation** replaces a number of randomly chosen bases by others; the number of mutations is equal

to a pre-set mutation rate. For **Survivor Selection**, the population and offspring are merged into a joint pool of individuals. These individuals are then sorted and the N individuals, least dominated by other individuals, are copied into the next generation.

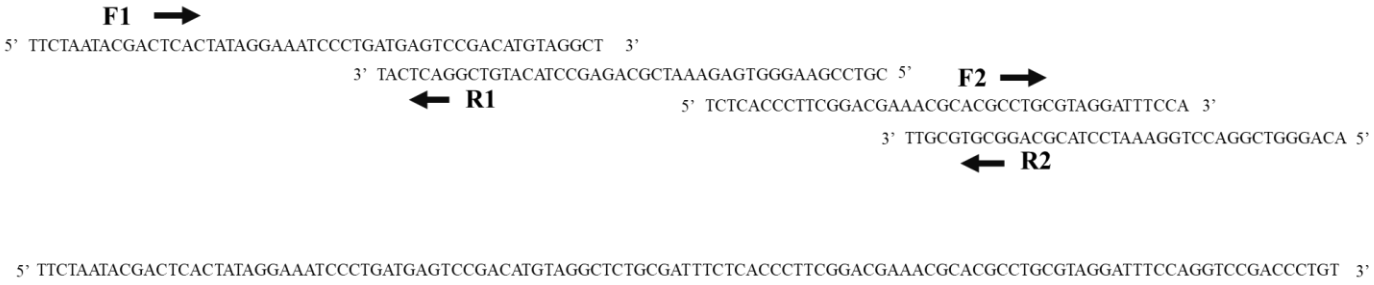

FIGURE S1. PCR Assembly

Primers F1, R1, F2 and R2 to generate full PCR template (shown at the bottom) of inducible ribozyme with T7 promoter sequence (Tian and Das 2017). All ribozymes (I -8, I-10, I-22 and Mn^{2+}-HHR) templates were generated using this protocol.

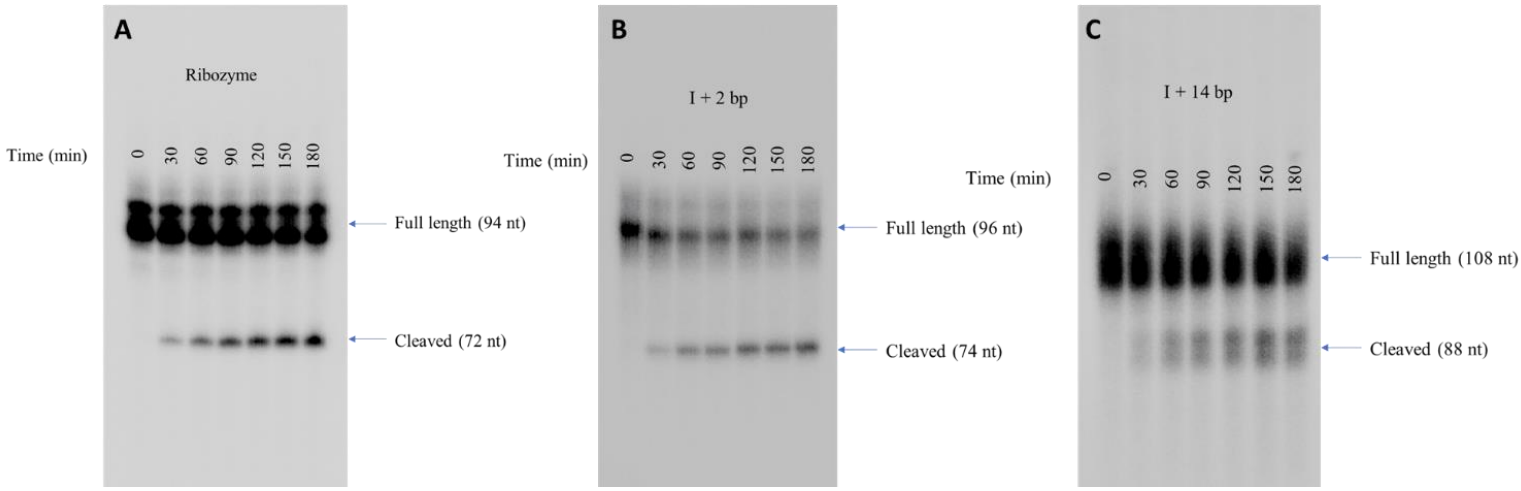

FIGURE S2. Ribozymes of different stem-I lengths cleavage assays

Ribozyme (A), I -2 bp (B) and I -14 bp (C) assay with standard cleavage conditions as mentioned in methods and materials section and products were separated on 10% denaturing polyacrylamide gel.

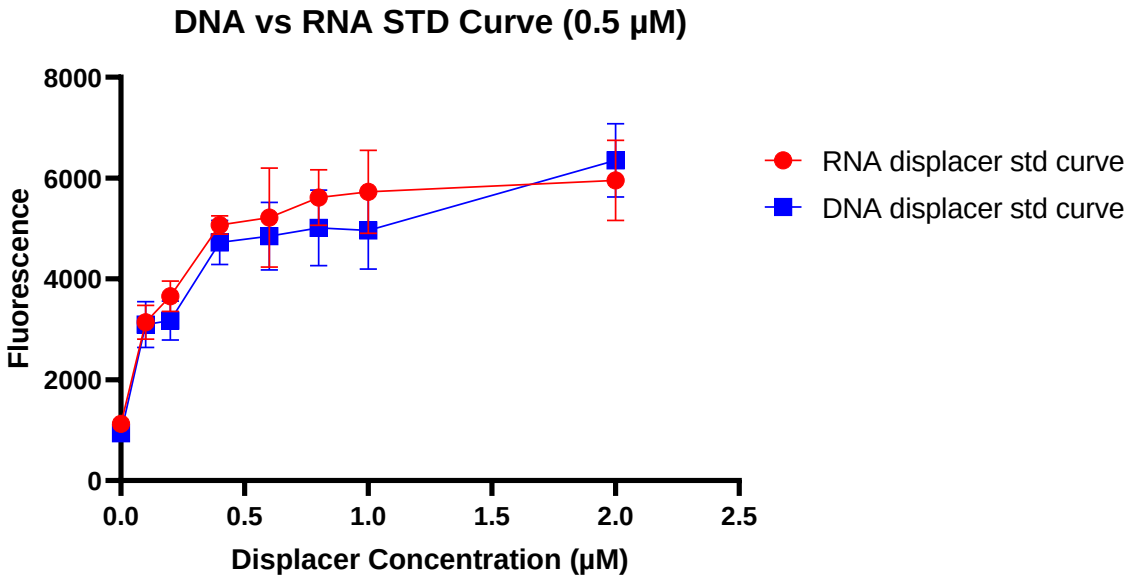

FIGURE S3. Standard curve comparison using DNA and RNA displacer

Standard curve comparison using DNA and RNA as displacer in same conditions and from same reaction mixture. This highlights that, at least with this sequence, DNA and RNA displacers have an equivalent displacing capacity. All reactions were incubated at 37°C for 15 minutes.

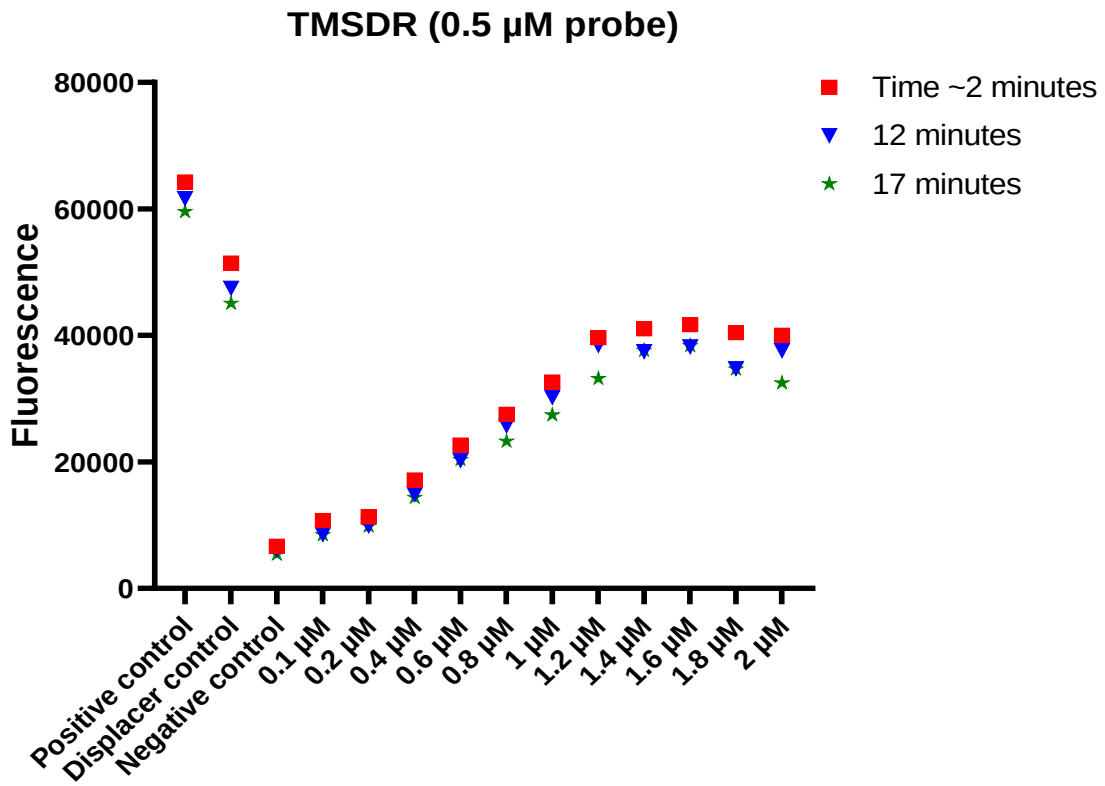

FIGURE S4. TMSDR assay using the DNA displacer.

TMSDR assay using the DNA displacer. Displacer/ probe ratio ranging from 0.2 to 4. Positive control comprised of fluorophore without quencher. Displacer control includes quenched probe with 10 µM of DNA displacer. Quenched probe was used as negative control. Time 0 is taken after inserting the plate into the plate reader (after ~2 mins). The well plate was incubated at 37°C inside the plate reader and the readings were taken at the described time points.

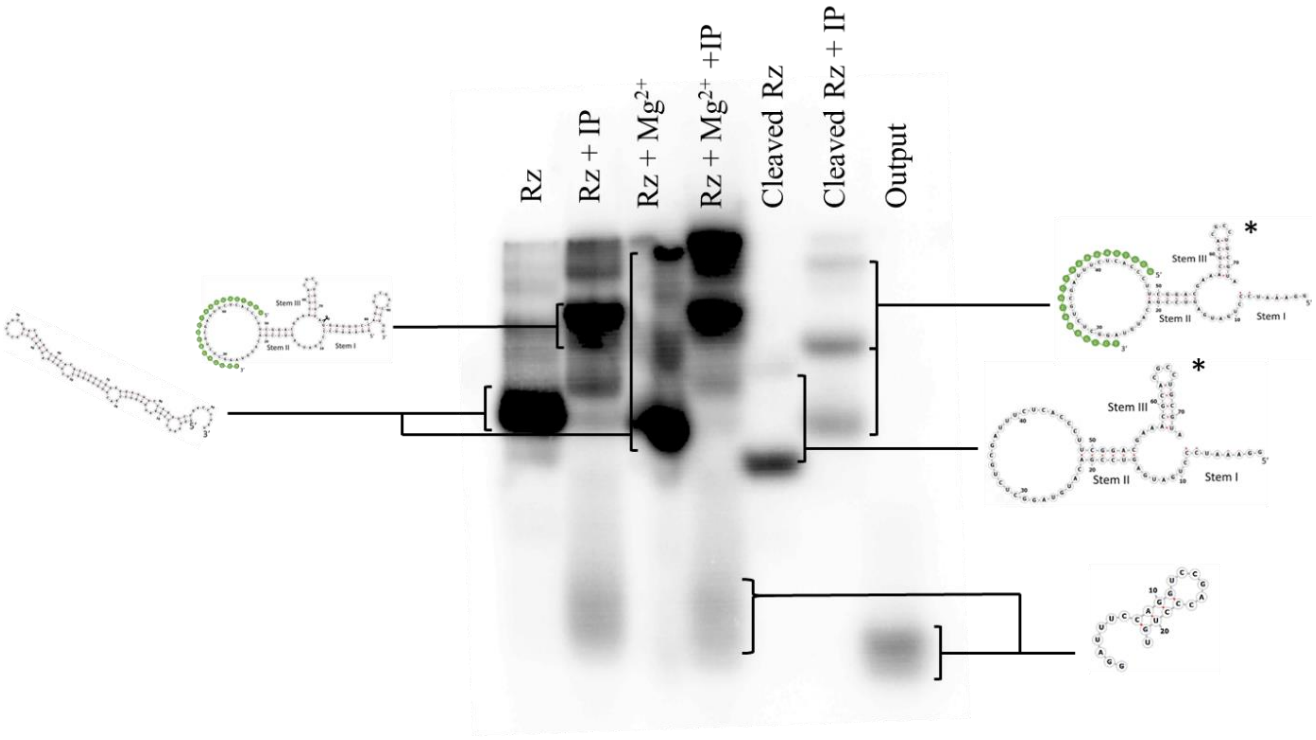

FIGURE S5. Native gel analysis of Ribozyme cleavage.

Ribozyme cleavage assay performed in standard conditions as mentioned in materials and methods. Lane as indicated, concentrations are 10 µM for Input and 10 mM for $MgCl_2$. Cleaved Ribozymes and Output lanes were done following initial purification of cleavage products on a denaturing (8 M urea) PAGE and then incubating these products in parallel with the "full length ribozyme samples". Asterisks mark cases which we hypothesize can produce higher order complexes (with multiple ribozymes and/or inputs), explaining why we see several bands in some lanes.

Moreover, it should be noted that cleavage can occur within the gel as it contains 5 mM Mg^{2+}, which can probably explain the presence of a large band corresponding to the output (21 bases product) in the lane "Rz +IP" (i.e. without Mg^{2+} in the reaction mix).

Materials and methods for native gel:

Native gel experiments were performed using 10% native polyacrylamide gel prepared in 1X TBMg (90 mM Tris base, 90 mM Boric acid, and 5 mM $Mg(OAc)_2$). Native gels were run at lower power (5W) to prevent heating. Running buffer was kept at 37°C to keep the temperature of the reaction constant from incubation to loading. Ribozyme cleavage assay performed in standard conditions as mentioned in materials and methods.

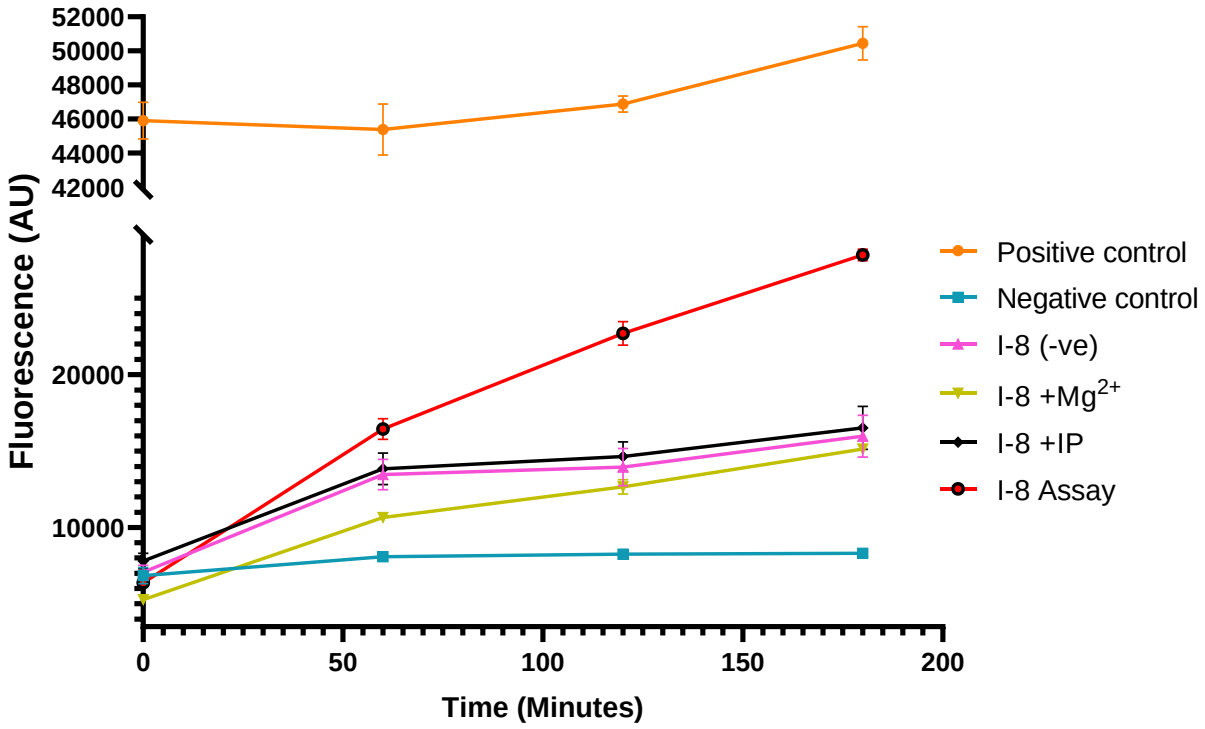

FIGURE S6. Ribozyme (stem I-8 bp) TMSDR assay with Mg^{2+} control.
Assay of HHR with a stem-I of 8 bp (I-8) denotes ribozyme with input oligo (10 µM) and Mg^{2+} (10 mM). The cleavage assay was performed in standard conditions as mentioned in materials and methods section: 1 µM of probe and 2 µM of ribozyme were used. 10 µM of the R-strand with the probe was used as positive control (i.e. a probe unquenched by a large excess of R-strand), and a quenched probe was used as negative control. Ribozyme without Mg^{2+} and without input DNA (I-8 (-ve); or with Mg^{2+} and without input DNA (I-8 + Mg^{2+}); or without Mg^{2+} and with input DNA (I-8 +IP); were used as HHR negative controls. The HHR assay (I-8 Assay) includes 10 µM input DNA and 10 mM Mg^{2+}. Readings were taken over a period of 180 minutes.

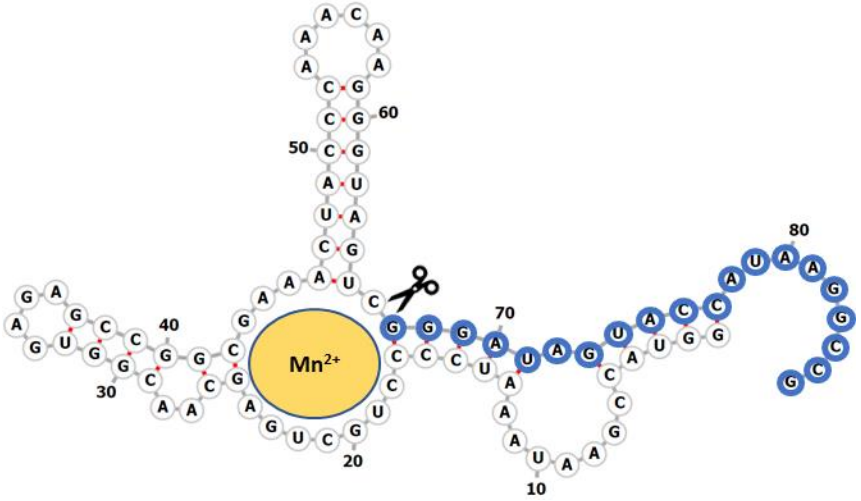

FIGURE S7. Schematic representation of Mn^{2+}-HHR with an alternative secondary structure

Schematic representation of Mn^{2+}-HHR with a secondary structure not taking pseudoknots in consideration. This alternative structure has more base pairs between the product and the ribozyme and would thus be expected to lead to slower release of the product compared to the structure in Figure 5A. Note also that both structures are likely to co-exist within the population of RNA structures in our assays.

Ribozyme with a stem I of 8 bp (I-8)	F1	5′TTCTAATACGACTCACTATAGGAAATCCCTGATGAGTCCGACATGTAG GCT 3′
	R1	5′CGTCCGAAGGGTGAGAAATCGCAGAGCCTACATGTCGGACTCAT 3′
	F2	5′TCTCACCCTTCGGACGAAACGCACGCCTGCGTAGGATTTCCA 3′
	R2	5′ACAGGGTCGGACCTGGAAATCCTACGCAGGCGTGCGTT 3′
	Input	5′GGGTGAGAAATCGCAGAGCCTA 3′
Ribozyme with a 10 bp stem I (I-10)	F1	5′TTCTAATACGACTCACTATACTGGAAATCCCTGATGAGTCCGACATGTA GGCT 3′
	R1	5′CGTCCGAAGGGTGAGAAATCGCAGAGCCTACATGTCGGACTCAT 3′
	F2	5′TCTCACCCTTCGGACGAAACGCACGCCTGCGTAGGA 3′
	R2	5′ACAGGGTCGGACCTGGAAATCCTACGCAGGCGTGCGTT 3′
Ribozyme with a 22 bp stem I (I-22)	F1	5′TTCTAATACGACTCACTATAACAGGGTCGGACCCTGGAAATCCCTGAT GAGTCCGACA 3′
	R1	5′TGAGAAATCGCAGAGCCTACATGTCGGACTCATCAGGGA 3′
	F2	5′AGGCTCTGCGATTTCTCACCCTTCGGACGAAACGCAC 3′
	R2	5′ ACAGGGTCGGACCTGGAAATCCTACGCAGGCGTGCGTTTCGTCCGAAG 3′
Mn^{2+}-HHR	F1	5′ TTCTAATACGACTCACTATAGGTACCGAATAAATCCCCTGCTGAGCAA 3′
	R1	5′CGCCGGCTCTCACCGTTGCTCAGCAGGGGATT 3′
	F2	5′GTGAGAGCCGGCGAAACTACCCAAACAAGGGTAGTCGGGATAGTACC AT 3′
	R2	5′CGGCCTTATGGTACTATCCCGACTACCCTTGT 3′

Table S1. Primerize (Tian and Das 2017) assembly for all generated ribozymes.

Ribozyme with a stem I of 8 bp	5′GGAAAUCCCUGAUGAGUCCGACAUGUAGGCUCUGCGAUUUCUCAC CCUUCGGACGAAACGCAGCCUGCGUAGGAUUUCCAGGUCCGACCCUG U 3′
Ribozyme with a 10 bp stem I (I+2bp)	5′CUGGAAAUCCCUGAUGAGUCCGACAUGUAGGCUCUGCGAUUUCUC ACCCUUCGGACGAAACGCACGCCUGCGUAGGAUUUCCAGGUCCGACC CUGU 3′
Ribozyme with a 22 bp stem I (I+14bp)	5′ACAGGGUCGGACCCUGGAAAUCCCUGAUGAGUCCGACAUGUAGGC UCUGCGAUUUCUCACCCUUCGGACGAAACGCACGCCUGCGUAGGAUU UCCAGGUCCGACCCUGU 3′
Mn^{2+} HHR	5′GGUACCGAAUAAAUCCCCUGCUGAGCAACGGUGAGAGCCGGCGAA ACUACCCAAACAAGGGUAGUCGGGAUAGUACCAUAAGGCCG 3′

Table S2. Ribozyme sequences

References

Tian S, Das R. 2017. Primerize-2D: automated primer design for RNA multidimensional chemical mapping. *Bioinformatics* **33**: 1405-1406.