Chapter 2

Cis-Acting 5' Hammerhead Ribozyme Optimization for In Vitro Transcription of Highly Structured RNAs

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Abstract

RNA-mediated biological processes usually require precise definition of 5′ and 3′ ends. RNA ends obtained by in vitro transcription using T7 RNA polymerase are often heterogeneous in length and sequence. An efficient strategy to overcome these drawbacks consists of inserting an RNA with known boundaries in between two ribozymes, usually a 5′ hammerhead and a 3′ hepatitis delta virus ribozymes, that cleave off the desired RNA. In practice, folding of the three RNAs challenges each other, potentially preventing thorough processing. Folding and cleavage of the 5′ hammerhead ribozyme relies on a sequence of nucleotides belonging to the central RNA making it more sensitive than the usual 3′ hepatitis delta virus ribozyme. The intrinsic stability of the central RNA may thus prevent correct processing of the full transcript. Here, we present a method in which incorporation of a full-length hammerhead ribozyme with a specific tertiary interaction prevents alternative folding with the lariat capping GIR1 ribozyme and enables complete cleavage in the course of the transcription. This strategy may be transposable for in vitro transcription of any highly structured RNA.

Key words Ribozymes, RNA folding, Lariat capping GIR1 ribozyme, In vitro transcription, RNA tertiary interactions

1 Introduction

To study the massive amount of RNAs identified by transcriptomic analysis, it is important to identify their boundaries in order to grab the "right" molecules. The history of ribozymes well illustrates this idea. At the beginning, the hammerhead ribozyme (HMH) was characterized in short versions that prevented determining the full extent of the catalytic properties of the natural versions [1]. Thus, biochemical studies showing that hammerhead ribozymes were orders of magnitude more efficient to fold and cleave under low magnesium concentrations if a tertiary interaction between stem I and the loop from stem II was occurring [2], came years later as a surprise (Fig. 1). This finding was further demonstrated by the crystal structure of the *Schistosoma* hammerhead ribozyme in which

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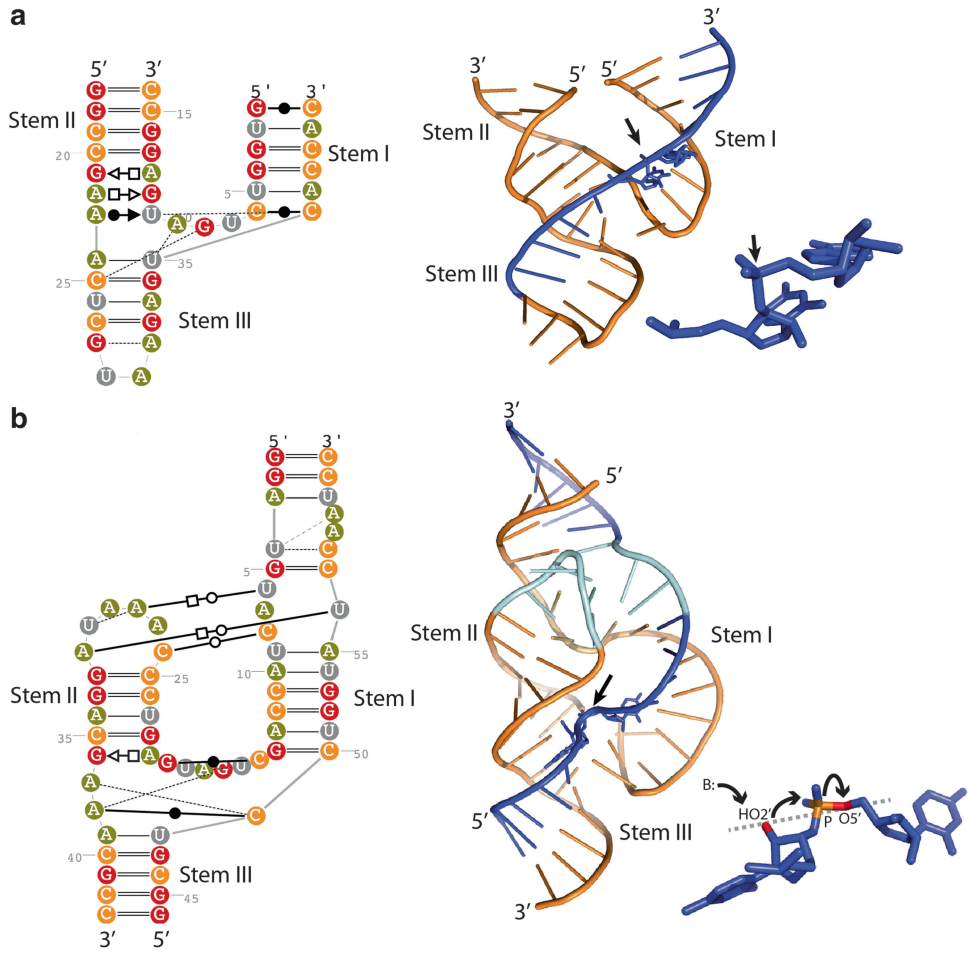

Fig. 1 Comparative secondary and three-dimensional structures of the hammerhead ribozyme cores from the minimal (**a**) versus the full-length (**b**) versions. Secondary structures (represented using the program Assemble [22]) indicate the sequence with nucleotides colored by type. For clarity, stem I is depicted with the 5′ strand on the *left*. Schemes in following figures depict the 5′ strand of stem I on the *right*. The crystal structures of the minimal [5] and full-length [3] hammerhead ribozymes present an *orange core* and a *blue substrate*. Nucleotides critical for tertiary interactions taking place in the full-length hammerhead are *colored in cyan* (**b**). The cleavage site is indicated with an *arrow*. The dinucleotides around the scissile bond are enlarged and displayed as *sticks*. The structures show the different conformations taking place in the two-ribozyme versions. A *dashed gray line* shows the alignment of the three colored atoms (O2′, P, O5′) involved in the in-line attack mechanism that takes place in the efficient version of the hammerhead ribozyme

setting the correct tertiary interaction leads to a shape of the catalytic site in which the chemical groups responsible for catalysis are ready for in-line attack [3]. This was not the case in crystal structures of the shorter versions [4, 5] (Fig. 1). Other examples like studies on the hairpin [6] and Varkud satellite [7] ribozymes also show that the originally defined molecules were not including all the determinants required for their full efficiencies, and further, the

characteristic modular architecture of RNA molecules [8]. The loss of structural integrity of important modules thus affects the overall RNA architecture, which in the end perturbs the function.

The potential occurrence of functional modules within the ends of RNA molecules justifies that they should be well defined for most studies. Unfortunately, the most widely used enzyme for in vitro production of RNA, the T7 RNA polymerase (T7RNAP), is prone to incorporate residues not encoded by the template at both the 5′ [9] and 3′ ends [10]. These uncoded residues eventually jeopardize experimental strategies such as crystallographic studies, dye-labeling for FRET studies, or in general any technique based on RNA ligation [11]. Moreover, any sequence immediately downstream from the promoter cannot be synthesized, since T7RNAP requires a specific G-rich starting sequence [12].

The errors made by T7RNAP can be concentrated in the tails of ribozymes located 5′ and 3′ of the RNA of interest that will be cleaved off [13]. This strategy can also be adapted to various systems including the production of RNAs with unfavorable 5′ sequences [14, 15], and/or to the use of ribozymes in *trans* [16]. On the 5′ side, a hammerhead ribozyme is usually chosen (Fig. 2). Only few nucleotides with any sequence downstream from the cleavage site are required for hybridization to fold the active ribozyme. On the 3′ side, the hepatitis delta virus (HδV) ribozyme is preferred because it is entirely located 3′ from the cleavage site and cleaves after any nucleotide, which leaves total freedom to the user. After cleavage, the central RNA is left with 5′ OH and 2′,3′ cyclic phosphate groups.

As long as the central RNA adopts a simple structure, this strategy does not need specific adaptation. However, if the structure of the central RNA is complex, it may interfere with proper folding of the flanking ribozymes and prevent efficient processing. While a short incubation at a temperature favoring structural rearrangements (~60 °C) usually restores cleavage by the 3′ HδV ribozyme, it is not the case for the 5′ hammerhead. This situation happened when we tried to express a lariat capping GIR1 ribozyme [17] with well-defined ends (Fig. 2). Nearly no cleavage was observed with a hammerhead version bearing a rather short stem I (5 bp). We assumed that the used truncated hammerhead had suboptimal activity because it could not form the critical tertiary interactions taking place between stem I and the loop of stem II. Our purpose was to design 5′ hammerhead constructs so that their substrate (stem I) resulted from hybridization to a longer sequence of the central lariat capping GIR1 ribozyme (LCrz) while preserving unpaired nucleotides, which interact with the facing loop from stem II (Fig. 3). This tertiary interaction, thereby preserved in the fusion between the hammerhead and the lariat capping GIR1 ribozymes, accelerates folding and catalysis and allows decreasing the required magnesium concentration [2].

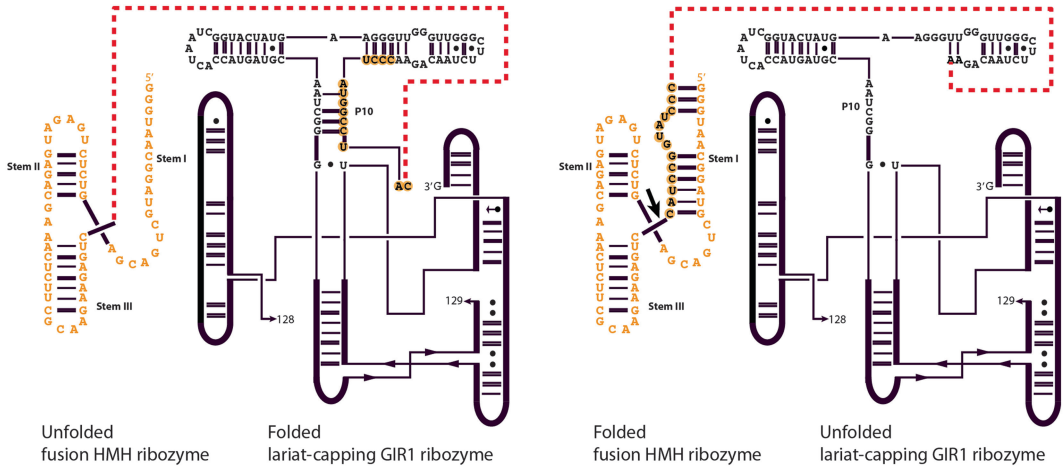

Fig. 2 Mutually exclusive theoretical secondary structures of the pre-RNA ribozyme transcript. If the pre-transcript presents an unfolded inactive hammerhead and a folded lariat-capping GIR1 ribozymes, the situation is unfavorable (*left panel*). However, the opposite situation (*right panel*) favors maximal cleavage during the in vitro transcription reaction. The bulge in stem I potentially forms a more stable structure with the loop closing stem II than if stem I is closed by a couple of additional A–U pairs. An *arrow* indicates the cleavage site

Design of the fusion hammerhead constructs using the program RNAfold [18] and their comparison to wild-type sequences of full-length hammerhead ribozymes was carried out. Selected hammerhead sequences with a stem I integrating the 5′ end of the lariat capping GIR1 ribozyme [17] were assayed for efficient cleavage in the course of in vitro transcription reactions (Fig. 4). The direct involvement of the bulge in stem I was investigated by determining the observed kinetic constants of the successful candidates (Fig. 5, [19]). Cloning of efficient fusion hammerhead ribozymes upstream from the lariat capping GIR1 ribozyme, followed by the HδV ribozyme, was performed in plasmid pUC19, further transcribed in vitro as linearized templates (Fig. 6). Northern blot analysis showed that the optimized hammerhead ribozymes were very efficient in the full context since they cleave to completion in the course of transcription, thus validating our design (Fig. 7). This method, validated on two different sequences of the lariat capping GIR1 ribozyme, is a practical addition to the molecular biologist's toolbox that addresses defining accurate ends to highly structured RNAs.

2 Materials

Prepare all solutions using Millipore or DEPC water. All buffers are filtered using a 0.22 μm sterile filter and kept at room temperature unless otherwise specified.

2.1 In Silico Design of Fusion Hammerhead Ribozyme Sequences

1. RNAfold [18] Web server can be used http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi.

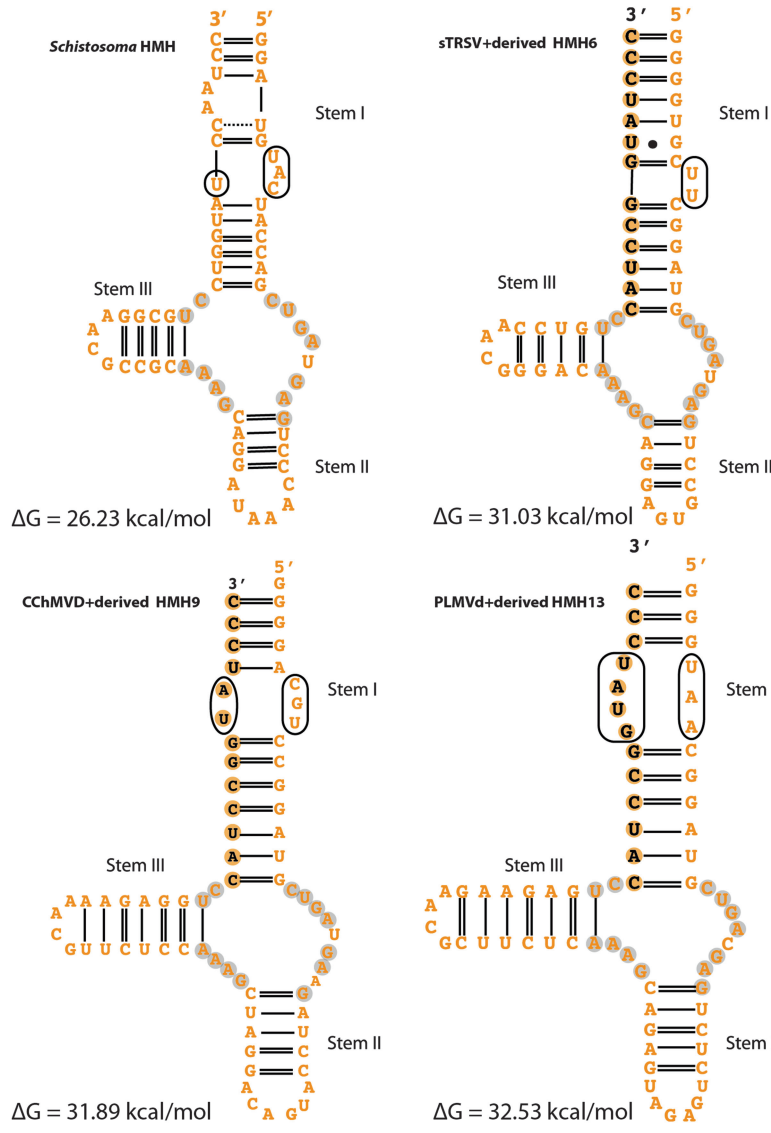

Fig. 3 Secondary structure diagrams of the fusion hammerhead ribozyme candidates (outputs from RNAfold) show a correct catalytic core structure and unpaired nucleotides in stem I. The secondary structure of the hammerhead ribozyme from *Schistosoma* is shown as a reference model with nucleotides from the bulge in stem I interacting with the *loop* from stem II *circled*. Some of the *circled* unpaired nucleotides in candidates HMH6, 9, and 13 potentially interact with the *loop* from stem II

Sequence of the full-length wild-type hammerhead ribozyme from *Schistosoma*: 5′ GGAUGUACUACCAGCUGAUGA GUCCCAAAUAGGACGAAAC GCCGCAAGGCGUCC UGGUAUCCAAUCC 3′.

Sequence of the full-length fusion hammerhead 6 ribozyme derived from sTRSV+: 5′ GGGGUGCUUCGGAUGC

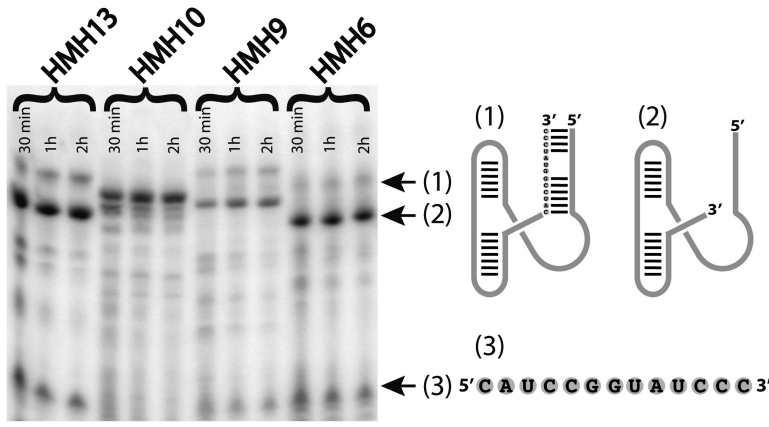

Fig. 4 Body-labeled RNA pattern from a standard transcription reaction of the full-length hammerhead ribozymes fused to the 13 nucleotides at the 5′ end of the lariat-capping GIR1 ribozyme. This step is meant to check whether ribozymes with the modified sequence, but lacking the main part of the lariat-capping GIR1 ribozyme, cleave in the course of the transcription reaction. The main RNA species numbered 1–3 are visible on the gel, (1) uncleaved hammerhead, (2) cleaved hammerhead core, (3) cleaved substrate (13 nt). HMH10, used as a negative control, carries a mutation ΔC15 with respect to the *Schistosoma* hammerhead ribozyme numbering (*see* Fig. 1b)

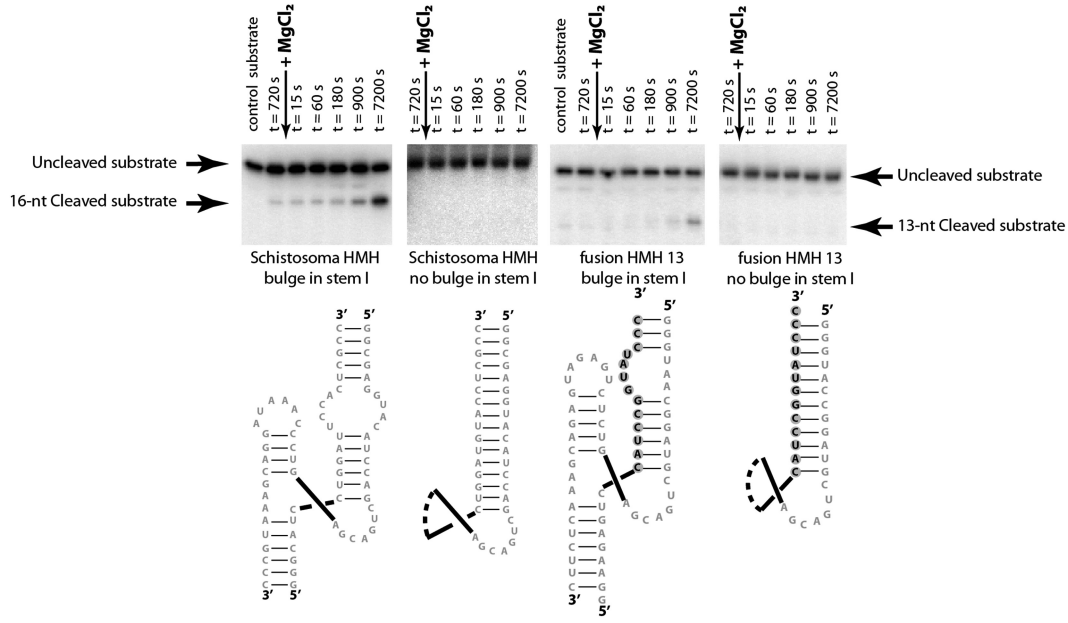

Fig. 5 Example of kinetic analysis of *trans* fusion hammerhead lariat capping GIR1 ribozymes. The model hammerhead from *Schistosoma* (*left panel*) is compared to HMH13 (*right panel*). A 50-fold excess of the in vitro transcribed hammerhead ribozyme core was added to the 3′ ^{32}P-labeled RNA substrate to achieve single turnover conditions. Two kinds of cores (and substrates in the case of the *Schistosoma* ribozyme) were used. The first kind preserved the bulge in stem I whereas the second kind forms a duplex with perfect complementarity. Cleavage is observed only when the bulge is present. The fraction of cleaved substrate was plotted versus time. Data were fitted to the equation $F_{cl} = F_{max}(1 - e^{-kt}) + F_0$ to determine k_{obs} values. The *Schistosoma* ribozyme presents a k_{obs} similar to HMH13, $k_{obsSch} = 0.013 \pm 4.7 \times 10^{-3}$ min^{-1} and $k_{obsHMH13} = 0.016 \pm 8.3 \times 10^{-3}$ min^{-1} showing that our designed hammerheads are as efficient as the *Schistosoma* model ribozyme at 0.1 mM $MgCl_2$

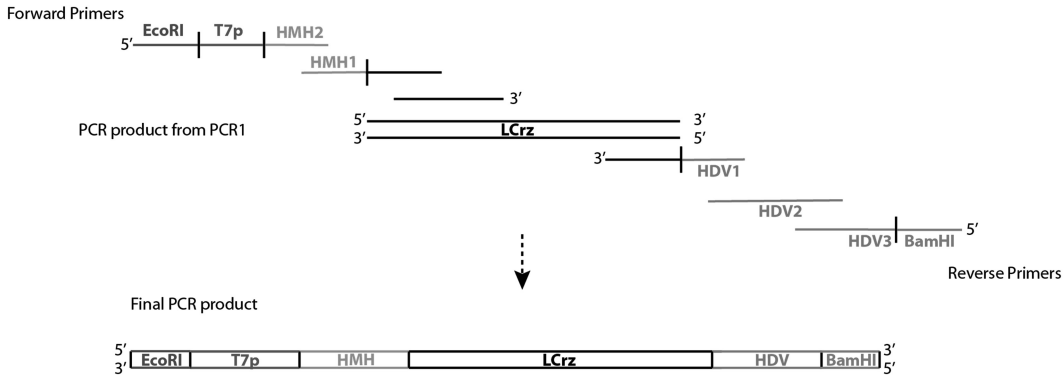

Fig. 6 Description of the PCR strategy of the DNA template encoding the ternary ribozyme system. A plasmid containing a 5′ truncated lariat-capping GIR1 ribozyme (LCrz) was used as a template for the first PCR amplification. Two further PCR steps created the flanking hammerhead and hepatitis δ virus (HδV) ribozymes, a T7 promoter and restriction sites for cloning (*Eco*RI, *Bam*HI)

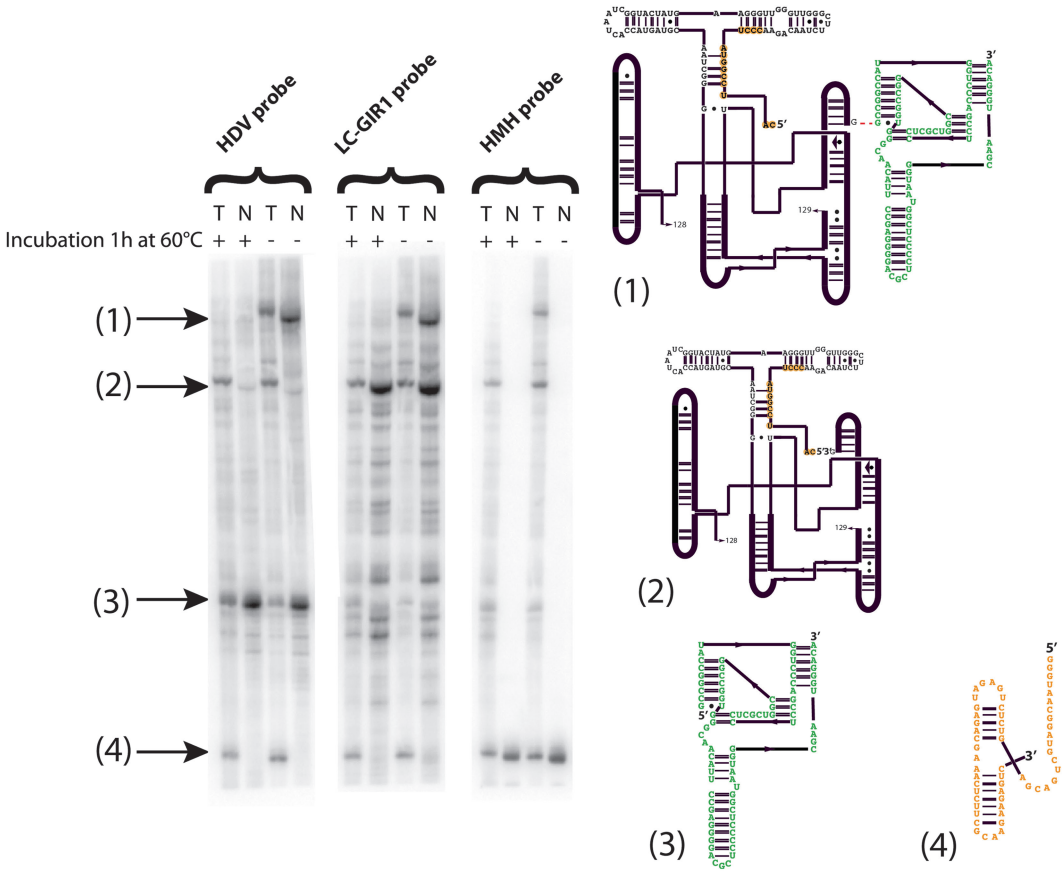

Fig. 7 Northern blot analysis of a transcription reaction of the ternary ribozyme construct. Body-labeled (T) and cold (N) transcription reactions were performed for 2 h and fractionated side by side by PAGE. Northern blot analysis was performed using a set of probes specific for (i) HδV, (ii) LCrz, and (iii) the hammerhead ribozymes. To complete HδV cleavage reactions, samples were incubated 1 h at 60 °C. Four main RNA products are visible on the membranes. (1) corresponds to the lariat-capping GIR1 ribozyme tethered to the HδV ribozyme. (2) is the correctly processed lariat-capping GIR1 ribozyme. (3) and (4) correspond to the two cleaved flanking ribozymes. It is important to note that cleavage by the hammerhead ribozyme goes to completion during the transcription reaction implying that the ternary ribozyme transcript cannot be detected

UGAUGAGUCCGUGAGGACGAAACAGGGCAA CCUGUCCAUCCGGUAUCCC 3′.

Sequence of the full-length fusion hammerhead 9 ribozyme derived from CChMVD+: 5′ GGGGACGUCCGGAUGC UGAUGAAGAUCCAUGACAGGAUCGAAAC CUCUUGCAAAAGAGGUCCAUCCGGUA UCCC 3′.

Sequence of the full-length ΔC15 fusion hammerhead 10 ribozyme derived from sLTSV–: 5′ GGGAGGUGCGG AUGAUGAGUCCGAAAGGACGAAACAGU AGGAAUACUGUCCAUCCGGUAUCCC 3′.

Sequence of the full-length fusion hammerhead 13 ribozyme derived from PLMVd+: 5′ GGGUAACGGAUGCUGAC GAGUCUCUGAGAUGAGACGAAACUCUUCG CAAGAAGAGUCCAUCCGGUAUCCC 3′.

2.2 In Vitro Determination of Fusion Hammerhead Ribozymes Cleavage Efficiencies

2.2.1 Co-transcriptional Cleavage of Fusion Hammerhead Ribozymes

1. DNA oligos are used as template for amplification by PCR creating the dsDNA template required for the in vitro transcription of the full-length hammerhead ribozymes from *Schistosoma* and full-length fusion hammerhead ribozymes HMH6, HMH9, HMH10, and HMH13.

   Forward (+) and reverse (–) strands for each hammerhead ribozyme:

   HMHSchistosoma+:5′TAATACGACTCACTATAGGATGTA CTACCAG CTGATGAGTCCCAAATAGGACGAAACGCCG CAAGGCGTCCTGGTATCCAATCC 3′.

   HMHSchistosoma–: 5′GGATTGGATACCAGGACGC CTTGC GGCGTTTCGTCCTATTTGGGACTCATCAGCT GGTAGTACATCCTATAGTGAGTCGTATTA 3′.

   HMH6+: 5′ TAATACGACTCACTATAGGGGTGCTTCGG ATGCTGATGAGTCCGTGAGGACGAAACAGGGCAA CCTGTCCATCCGGTATCCC 3′.

   HMH6–: 5′ GGGATACCGGATGGACAGGTTGCCCTGT TTCGTCCTCACGGACTCATCAGCATCCG AAGCACCCCTATAGTGAGTCGTATTA 3′.

   HMH9+: 5′ TAATACGACTCACTATAGGGGACGT CCG GATGCTGATGAAGATCCATGACAGGATCGAAA CCTCTTGCAAAAGAGGTCCATCCGGTATCCC 3′.

   HMH9–: 5′ GGGATACCGGATGGACCTCTTTTGCAAGA GGTTTCGATCCTGTCATGGATCTTCATC AGCATCCGGACGTCCCCTATAGTGAGTCGTATTA 3′.

   HMH10+: 5′ TAATACGACTCACTATAGGGAGGTGCGG ATGATGAGTCCGAAAGGACGAAACAGTAGG AATACTGTCCATCCGGTATCCC 3′.

HMH10–: 5′ GGGATACCGGATGGACAGTATTCCTATG TTTCGTCCTTTCGGACTCATCATCCGCACCTCCCTA TA GTGAGTCGTATTA 3′.

HMH13+:5′TAATACGACTCACTATAGGGTaaCGGATGC TGACGAGTCTCTGAGATGAGACGAAACTCTTCGC AAGAAGAGTCCAT CCGGTATCCC 3′.

HMH13–: 5′ GGGATACCGGATGGACTCTTCTTGCGAA GAGTTTCGTCTCATCTCAGAGACTCGTCAGCA TCCGTTACCCTATAGTGAGTCGTATTA 3′.

2. DNA primers used for PCR amplification of the dsDNA template:

Primer 1: 5′ TAATACGACTCACTATA 3′.

Primer 2 for fusion hammerhead ribozymes: 5′ GGGATACCGG ATGGAC 3′.

Primer 2 for hammerhead from *Schistosoma*: 5′ GGATTGGATA CCAG 3′.

3. Phusion High-Fidelity DNA polymerase (2,000 U/mL) stored at –20 °C.
4. 5× Phusion High-Fidelity DNA polymerase HF reaction buffer stored at –20 °C.
5. 25 mM dNTPs mix stock solution is stored at –20 °C.
6. 6× glycerol blue loading buffer: 50 % glycerol, 0.1 % xylene cyanol, 0.1 % bromophenol blue.
7. 2 % (w/v) agarose gel: agarose for routine use is dissolved in 100 mL of 1× TBE.
8. Steady red [α-^{32}P]-UTP at a concentration of 10 mCi/mL and a specific activity of 3,000 Ci/mmol is stored at 4 °C.
9. Micro Bio-Spin 6 chromatography columns (Bio-Rad), or comparable product.
10. His-tag-T7 RNA polymerase is produced in the laboratory from plasmid pT7-911Q [20].
11. 5× TMSDT buffer: 200 mM Tris–HCl, pH 8.1, 110 mM $MgCl_2$, 5 mM spermidine, 25 mM DTT, 0.05 % Triton stored at –20 °C.
12. Alkaline phosphatase is stored at a concentration of 1 U/μL in 1× TMSDT at 4 °C.
13. 100 mM ATP, 100 mM UTP, 100 mM CTP, and 100 mM GTP stock solution are stored at –20 °C.
14. 2× loading buffer: 8 M urea, 0.025 % xylene cyanol, 0.025 % bromophenol blue.
15. 10× Tris-borate-EDTA buffer (TBE) to run PAGE: 0.89 M Tris base, 0.89 M boric acid, 20 mM EDTA, pH 8.0.

16. Denaturing PAGE: 10 % acrylamide/bisacrylamide (19:1), 8 M urea in 1× TBE.
17. Gel size: 35 cm width × 42 cm length × 0.08 cm thickness.
18. Apparatus to vacuum dry gels.
19. 35 × 43 cm imaging plate (FujiFilm), or comparable product.
20. Bio-imager (FujiFilm), or comparable equipment.

2.2.2 Kinetic Analysis of Fusion Hammerhead Ribozymes with Trans substrates

1. Substrate RNAs were purchased (e.g., at Dharmacon, Thermo Scientific).
2. Shaker MixMate (Eppendorf), or comparable equipment.
3. 3 M KCl stock solution.
4. T4 RNA ligase (5 U/μL) is stored at –20 °C.
5. 10× T4 RNA ligase reaction buffer: 500 mM Tris–HCl, pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP stored at –20 °C.
6. Steady red ^{32}pCp at a concentration of 10 mCi/mL and a specific activity of 3,000 Ci/mmol is stored at 4 °C.
7. X-ray autoradiograms (e.g., Super RX from Fuji Medical).
8. 10× kinetic buffer: 500 mM Tris–HCl, pH 8.0, 1 M NaCl, 1 mM EDTA, pH 8.0.
9. 1 M $MgCl_2$ stock solution.
10. 2× formamide-EDTA blue: 80 % formamide, 50 mM EDTA, pH 8.0, 0.02 % bromophenol blue.
11. Science Lab 2003 Image Gauge 4.2 (FujiFilm), or comparable quantification software.
12. KaleidaGraph 4.02, or comparable software for curve fitting.

2.3 Cloning of Ternary Ribozyme Constructs

1. DNA oligos used to create the insert corresponding to the ternary ribozyme constructs to be cloned:

   Forward primers:

   EcoRI_T7p_HMH2: 5′ GACGGCCAGTGAATTCTAA TACGACTCACTATAGGGTAACGGATGCTGACGAGT CTCTGAGA 3′.

   HMH1_LCrz: 5′ CTGACGAGTCTCTGAGATGA GACGA AACTCTTCGCAAGAAGAGTCCATCCGGTATCCCAA GA 3′.

   Reverse primers:

   BamHI_HδV3: 5′ GGATCCTCTAGAGTCGACCTTGTCC CATTCGCCATTACCGAGGGGACGGTCCCCTCGG AATGTT 3′.

   HδV2: 5′ GTCCCCTCGGAATGTTGCCCACCGGCCGCC AGCGAGGAGGCTGGGACCATGGCCGGC 3′.

HδV1_LCrz: 5′ GGACCATGGCCGGCGATTGTCTT GGGATACCGGATGCTTCCTTTCGG AACGACT 3′.

2. Gel-extraction kit.
3. pUC19 vector stored at a concentration of 1 mg/mL at –20 °C.
4. Cloning kit.
5. Miniprep spin kit.

2.4 Northern Blot Analysis of the Cleavage Pattern of the Ternary Ribozyme Transcripts

1. DNA probes for Northern blot:
   HMH: 5′ GACTCTTCTTGCGAAGAGTTTC 3′.
   LCrz: 5′ CATGATACTTCCCAACCCAACC 3′.
   HδV: 5′ CTCGGAATGTTGCCCACCGGCC 3′.
2. Steady red [γ-^{32}P]-ATP at a concentration of 10 mCi/mL and a specific activity of 3,000 Ci/mmol is stored at 4 °C.
3. T4 polynucleotide kinase (PNK; 10 U/μL). Store at –20 °C.
4. 10× PNK buffer: 500 mM Tris–HCl, pH 7.6, 100 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine. Store at –20 °C.
5. Nylon membrane for Northern blot analysis.
6. Stratalinker UV cross-linker (Stratagene).
7. Hybridization bottle: 35 × 300 mm.
8. 6× SSPE: 3 M NaCl, 0.2 M NaH_2PO_4, and 0.02 M EDTA. pH is adjusted to 7.4 with NaOH.
9. Pre-hybridization buffer: 1× SSPE, 2.5 % SDS, 2.5 % Denhart. Pre-hybridization buffer is stored at 30 °C to avoid SDS precipitation.
10. 50× Denhart's solution: 1 % (w/v) ficoll 400, 1 % (w/v) polyvinylpyrrolidone, 1 % (w/v) bovine serum albumin (fraction V). Denhart's solution should be freshly made.
11. Hybridization incubator.

3 Methods

3.1 In Silico Design of Fusion Hammerhead Ribozyme Sequences

The aim of this step is to design fusion hammerhead ribozymes presenting a hammerhead catalytic core fused to a substrate stem corresponding to the 5′ sequence of the lariat capping GIR1 ribozyme. The ribozyme strand presents both ends rooted in stem I, while loops close stems II and III. Stems II and III and the single-stranded regions form the catalytic core are directly copied from genuine hammerhead sequences presenting tertiary contacts between stem I and the loop of stem II (sTRSV+, CChMVD+, PLMVD+, [2]). Stem I should be approximately a dozen nucleotides long with a 3′ strand being the 5′ end of the central RNA, the lariat-capping GIR1 ribozyme in this case [17]. Consequently the

5′ strand of stem I is designed to be partially complementary to the latter and starts with three G residues for transcriptional efficiency (Fig. 2). Moreover, a bulge intervening six nucleotides after the cleavage site has to be preserved in stem I. The purpose of this bulge is to avoid formation of a perfect duplex which would destroy the tertiary contacts with the loop of stem II (Fig. 1) that are crucial for efficient folding and cleavage of the ribozyme (Fig. 3, [19]). In the sequences resulting from the fusion between hammerhead ribozymes and the lariat-capping GIR1 ribozyme, the presence of (1) a correct three-way junction forming the catalytic core and of (2) the critical unpaired nucleotides in stem I was systematically checked using RNAfold [18] (*see* **Note 1**).

3.2 In Vitro Determination of Fusion Hammerhead Ribozymes Cleavage Efficiencies

Successful sequences were transcribed from commercial template DNA oligos. The extent of cleavage was monitored in the course of the transcription reactions (Fig. 4). More detailed kinetic analysis of the most efficient transcripts, HMH13, were performed under single turnover conditions using fusion hammerhead ribozymes consisting of distinct catalytic and substrate strands (Fig. 5).

3.2.1 Co-transcriptional Cleavage of Fusion Hammerhead Ribozymes

To produce RNAs corresponding to candidate sequences selected in silico by in vitro transcription [12, 21], DNA templates preceded by a Class III T7 promoter (5′ TAA TAC GAC TCA CTA TA 3′) should be produced (Fig. 4).

1. Design full-length template DNA oligos, forward and reverse strands that will be used as dsDNA templates for in vitro transcription of the candidates sequences selected in silico (*see* **Note 2**).
2. Design primers for PCR amplification of the template DNA oligos (*see* **Note 3**).
3. Prepare 50 µL of PCR reaction containing: 300 ng of each DNA template, 0.5 µL of the Phusion High-Fidelity DNA polymerase (2,000 U/mL), 10 µL of the 5× Phusion HF buffer, 1.2 µM of forward primer, 1.2 µM of reverse primer, 400 µM dNTPs mix, water to 50 µL.
4. Run the PCR reaction in a thermal cycler following the set up displayed in Table 1.
5. Check the efficiency of amplification by mixing 5 µL of the PCR reaction to 1 µL 6× glycerol blue loading buffer and load onto a 2 % (w/v) agarose gel.
6. After ethidium bromide staining, observe the DNA migration pattern under UV.
7. Clean the PCR reaction using the Micro Bio-Spin columns.
8. Set up 50 µL in vitro transcription reactions containing: 1 µg of the cleaned PCR reaction, 10 µL of 5× TMSDT buffer, 5 mM ATP, 5 mM GTP, 5 mM CTP, 2.5 mM UTP, 25 µCi

Table 1
PCR program

Step	Temperature (°C)	Time	Number of cycles
Initial denaturing	95	3 min	1
Denaturing	95	30 s	30
Annealing	45[a]/50[b]	1 min	
Elongation	72	1 min	
End	4	10 min	1

[a]Annealing temperature for PCR of **step 4** in Subheading 3.2.1
[b]Annealing temperature for PCR of **step 3** in Subheading 3.3

[α-^{32}P]-UTP (10 mCi/mL, 3,000 Ci/mmol), 0.2 μL of alkaline phosphatase (1 U/μL) (*see* **Note 4**), T7 RNA polymerase as indicated by your provider (*see* **Note 5**), water to 50 μL.

9. Incubate at 37 °C for 2 h.
10. Remove a 12 μL aliquot of each transcription reaction after 30 min, 1 h and 2 h.
11. Stop the transcription by mixing the sample to an equal volume of 2× loading buffer.
12. Clean the transcription reaction using the Micro Bio-Spin columns (*see* **Note 6**).
13. Fractionate each sample by 10 % denaturing PAGE. Run the gel for 2 h at 60 W (*see* **Note 7**).
14. Vacuum dry the gel onto Whatman 3MM paper for 4 h at 80 °C or alternatively seal it in a plastic film and store it at −80 °C.
15. Expose the gel to an imaging plate in a cassette for 10 min (*see* **Note 8**).
16. Read the imaging plate using a Bio-imager.

3.2.2 Kinetic Analysis of Fusion Hammerhead Ribozymes with Trans substrates

This step can be performed as in [19] to check whether the bulge in stem I is functional or to determine an optimal magnesium concentration for cleavage (*see* **Note 9**). The hammerhead cores are obtained by in vitro transcription of dsDNA templates while substrates are obtained by RNA solid phase chemical synthesis (Fig. 5).

1. Design two sets of fusion hammerhead ribozymes and their substrates as separate RNA molecules. From this step the catalytic cores of the fusion hammerhead ribozymes will be handled separately from their substrate domains.
2. Purify the complementary DNA oligos that will form the dsDNA templates used for in vitro transcription of the different ribozymes' cores by 10 % denaturing PAGE.

3. Mix 20 µg of the forward and the reverse pure template DNA oligos and add KCl up to a final concentration of 500 mM. To hybridize the template DNA oligos heat the sample for 1 min at 95 °C and allow it to cool to room temperature for 10 min.
4. Set up 200 µL transcription reactions containing 40 µg of dsDNA template, 40 µL of 5× TMSDT buffer, 5 mM ATP, 5 mM GTP, 5 mM CTP, 5 mM UTP, 0.8 µL of alkaline phosphatase (1 U/µL) (*see* **Note 4**), T7 RNA polymerase as indicated by your provider (*see* **Note 5**), water to 200 µL.
5. Purify transcription reactions by 10 % denaturing PAGE.
6. Identify RNA bands corresponding to the fusion hammerhead core by UV shadowing.
7. Cut the band of interest out of the gel and place it into a 1.5 mL tube.
8. Immerse the gel band in about 800 µL of water and place the tube in a shaker overnight at 4 °C.
9. Decant the eluate and transfer it to a fresh tube.
10. Recover the fusion hammerhead cores by ethanol precipitation of the eluate.
11. Set up a 3′ radio-labeling reaction for the RNA substrates: 500 ng of RNA substrate, 40 µCi ^{32}pCp (10 mCi/mL, 3,000 Ci/mmol), 10 % DMSO, 1 µL T4 RNA ligase (5 U/µL), 3 µL 10× T4 RNA ligase buffer, water to 30 µL.
12. Incubate at 16 °C overnight.
13. Clean the labeling reactions using the Micro Bio-Spin columns (*see* **Note 6**).
14. Purify the labeled RNA substrates by 20 % denaturing PAGE at 50 W for 2 h.
15. Localize the positions of the labeled RNAs using an X-ray sensitive film.
16. Repeat **steps 7–10**.
17. Set up a reaction for analyzing the kinetics of the hammerhead cleavage under single turnover conditions as follows: 0.5 µM of purified fusion hammerhead core RNA, 10 nM of the purified 3′ radio-labeled substrate RNA, 1.5 µL of 10× kinetic buffer, water to 13 µL.
18. Incubate the reaction at 70 °C for 2 min and then at 25 °C for 10 min.
19. Take a 2 µL aliquot of the reaction.
20. Add $MgCl_2$ to a final concentration of 0.1 mM.
21. Incubate the reaction at 25 °C. Take a 2 µL aliquot of the reaction after 15 s, 1 min, 3 min, 15 min, 2 h.

22. Quench the reaction by mixing the aliquot with 2 μL 2× formamide-EDTA blue.
23. Place samples in ethanol cooled down by dry ice.
24. Separate the cleaved from uncleaved substrates by 12 % denaturing PAGE at 50 W for 2 h (*see* **Note 7**).
25. Vacuum dry the gel onto Whatman 3 MM paper for 4 h at 80 °C or alternatively seal it in a plastic film and store it at −80 °C.
26. Expose the gel to an imaging plate overnight in a cassette (*see* **Note 10**).
27. Reveal the radioactivity pattern on the imaging plate using a Bio-imager.
28. Quantify the amount of cleaved substrate using appropriate software.
29. Plot the fraction of substrate cleaved versus time and determine k_{obs} by fitting the data to the equation, $F_{cl} = F_{max}(1 - e^{-kt}) + F_0$ using an appropriate software like KaleidaGraph (*see* **Note 11**).

3.3 Cloning of Ternary Ribozyme Constructs

The most efficient fusion hammerhead ribozyme is cloned upstream from the lariat capping GIR1 ribozyme followed by an HδV ribozyme ending with a restriction endonuclease site used for linearization to permit run off transcription. Since cloning is the best choice for storing matrices in the laboratory's library, it is advised to anticipate the cloning step at the very beginning of the sequence design by adding a 5′ sequence either corresponding to the desired restriction enzyme recognition sequence or to the recombination sequence specified in the cloning kit.

All lariat capping GIR1 RNA constructs from *Didymium iridis* (DiGIR1) were transcribed from plasmid DNA templates obtained by cloning of an insert generated by three successive PCR amplifications. The 5′ PCR primers were designed to contain an *Eco*RI restriction site, a T7 promoter, the sequence of the co-transcriptional self-cleaving hammerhead ribozyme, and the 5′ sequence of the DiGIR1 ribozyme. The 3′ PCR primers included a *Bam*HI restriction site, a posttranscriptional self-cleaving HδV ribozyme, and the 3′ sequence of the lariat capping DiGIR1 ribozyme (Fig. 6).

1. Design forward and reverse primers for the successive PCRs (*see* **Note 12**).
2. Prepare 50 μL of PCR reaction containing: 50 pg of template plasmid DNA, 0.5 μL of the Phusion High-Fidelity DNA Polymerase (2,000 U/mL), 10 μL of the 5× Phusion HF buffer, 1.2 μM of forward primer, 1.2 μM of reverse primer, 400 μM dNTP mixture, water to 50 μL (*see* **Note 13**).

3. Run the PCR reaction in a thermal cycler following the set up displayed in Table 1 (*see* **Note 14**).
4. Mix the PCR reaction to 10 µL 6× glycerol blue loading buffer and load the entire PCR reaction onto a 2 % (w/v) agarose gel.
5. After ethidium bromide staining, observe the DNA migration pattern under UV.
6. Cut the DNA band of interest out of the gel.
7. Recover the DNA insert in 30 µL of water with a gel-extraction kit (*see* **Note 15**).
8. Insert the purified PCR product into a pUC19 plasmid using a cloning kit.
9. Directly transform XL-1 blue *Escherichia coli* competent cells with the recombinant plasmid product (*see* **Note 16**).
10. Grow the transformed XL-1 blue *E. coli* competent cells on LB/Ampicillin (100 µg/mL) plates overnight at 37 °C.
11. Grow 3 mL of LB/Ampicillin (100 µg/mL) cultures from individual colonies.
12. Extract DNA using a miniprep spin kit.
13. Sequence the extracted DNA using the M13 sequencing primers.
14. Prepare glycerol stocks of the clones containing the desired sequences by mixing 1 mL of saturated bacterial culture to 600 µL of sterilized 50 % glycerol solution in 2 mL screw cap tubes. Store the glycerol stocks at −80 °C.

3.4 In Vitro Transcription of the Ternary Ribozyme RNA Using Linearized Plasmids as Templates

The transcription reaction of the plasmid template is performed as for the PCR template except that the plasmid is linearized prior to the reaction and that the mass added is greater, since the molarity of the template is important.

1. For in vitro transcription, linearize the plasmid with *Bam*HI restriction endonuclease following the enzyme provider instructions.
2. Set up 50 µL in vitro transcription reactions containing: 2.5 µg of linearized plasmid DNA, 10 µL of 5× TMSDT buffer, 5 mM ATP, 5 mM GTP, 5 mM CTP, 5 mM UTP, 0.2 µL of alkaline phosphatase (1 U/µL) (*see* **Note 4**), T7 RNA polymerase as indicated by your provider (*see* **Note 5**), water to 50 µL.
3. In parallel set up 50 µL in vitro transcription reactions containing: 2.5 µg of linearized plasmid DNA, 10 µL of 5× TMSDT buffer, 5 mM ATP, 5 mM GTP, 5 mM CTP, 2.5 mM UTP, 25 µCi [α-^{32}P]-UTP (10 mCi/mL, 3,000 Ci/mmol), 0.2 µL of alkaline phosphatase (1 U/µL) (*see* **Note 4**), T7 RNA polymerase as indicated by your provider (*see* **Note 5**), water to 50 µL.

4. Incubate at 37 °C for 2 h.
5. Incubate half of each transcription at 60 °C for 1 h.
6. Stop transcription reaction by mixing it to an equal volume of 2× formamide-EDTA blue loading buffer.

3.5 Northern Blot Analysis of the Cleavage Pattern of the Ternary Ribozyme RNA (See Fig. 7)

1. Design three DNA probes that will be used to hybridize specifically either to the hammerhead ribozyme or to the central RNA or to the HδV ribozyme (*see* **Note 17**).
2. Label each DNA probe by mixing: 1 μM of DNA oligo, 2.5 μL of [γ-^{32}P]-ATP (10 mCi/mL, 3,000 Ci/mmol), 1 μL of T4 polynucleotide kinase (10 U/μL), 5 μL 10× PNK buffer, water to 50 μL.
3. Incubate 30 min at 37 °C, then 10 min at 65 °C.
4. Clean the labeling reactions using the Micro Bio-Spin columns (*see* **Note 6**).
5. Fractionate samples from in vitro transcription reactions of the ternary ribozyme RNA by 8 % denaturing PAGE. Load in four consecutive lanes the 60 °C pre-incubated or not samples in order to be able to compare the cold to the corresponding body-labeled transcription reactions. Repeat the same operation two times. Each set of four lanes will be used for a different DNA probe. Run for 3 h at 60 W (*see* **Note 7**).
6. Transfer the RNA bands from the gel onto positively charged nylon membrane using a vacuum gel dryer for 4 h at 80 °C.
7. Wash the membrane in water to remove the dried gel particles (*see* **Note 18**).
8. Cross-link the RNA onto the membrane using a UV cross-linker.
9. Cut the membrane to separate each set of four lanes. From this step you will be dealing with three membranes in parallel.
10. Insert delicately each membrane into a hybridization bottle (*see* **Note 19**).
11. Add 20 mL of pre-hybridization buffer.
12. Incubate at 60 °C for 30 min in a rolling hybridization oven.
13. Add the total amount of one labeled DNA primer per hybridization bottle.
14. Incubate at 37 °C over night in the rolling hybridization oven.
15. Wash each membrane with 20 mL of 6× SSPE at 37 °C for 5 min in the rolling hybridization oven. Repeat this step three times.
16. Wash each membrane with 20 mL of 6× SSPE at 42 °C for 5 min in the rolling hybridization oven.
17. Seal each membrane in a plastic film.

18. Expose an imaging plate to the plastic sealed membrane for 3 h in a cassette (*see* **Notes 8** and **10**).
19. Reveal the radioactivity pattern on the imaging plate using a Bio-imager.

4 Notes

1. The sequence of the adapted substrate starts after the cleavage site and preserves several unpaired residues in stem I of the ribozyme core to favor tertiary contacts with the loop of stem II. The best situation is when the UAC sequence forms a bulge. When this not possible, avoid building an extended helix by inserting at least non-complementary residues.
2. Do not forget to insert the T7 promoter sequence upstream of the DNA templates.
3. These primers can be the same for all fusion hammerhead ribozymes candidates if you use as primer 1 the sequence of the T7 promoter and as primer 2 the sequence of the central RNA.
4. It is not compulsory to add alkaline phosphatase but it is known to increase in vitro transcription yields.
5. We produce our own T7 RNA polymerase, which reduces cost considerably.
6. It is not mandatory to clean transcription reaction although it is preferable to remove not incorporated radio-labeled nucleotides to avoid contamination of the electrophoresis buffer.
7. To have better defined bands, gels are pre-run for 1 h at 60 W.
8. The radioactivity pattern of the gel can as well be obtained on X-ray autoradiogram.
9. This step is not mandatory and was carried out as a proof of principle. It can be performed if the ribozyme construct is inactive to further check for efficiency of a set of hammerhead constructs carrying different kinds of bulges in stem I.
10. Exposure time of the imaging plate depends on the amount of ^{32}P-labeled material on the membrane or gel.
11. F_{cl} is the fraction of cleaved substrate, F_0 is the initial amount of substrate cleaved before addition of $MgCl_2$, F_{max} is the maximal amount of substrate cleaved, t is the time in minute and k_{obs} is the observed rate of cleavage.
12. For optimal yield, forward and reverse primers should have close melting temperature.
13. Keep your samples on ice while preparing the PCR reaction.
14. Insert your samples in the thermal cycler when the temperature of the sample holder is at 90 °C minimum.

15. To avoid any troubles with next steps of the cloning process, 10 % of 3 M ammonium acetate, pH 5.2, is added to the recovered DNA and precipitated with 3 volumes of pure ethanol. DNA is then resuspended in 30 μL of water.
16. Very efficient chemically competent *E. coli* cells can be prepared if all the material is maintained ice-cold throughout the whole preparation process.
17. The three primers should have close melting temperature and the melting temperature must be above 42 °C.
18. Be delicate not to scratch the membrane.
19. Be careful that the side of the membrane on which the RNA is cross-linked is facing the inside of the hybridization bottle. There should not be any membrane overlap in the hybridization bottle.

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References

1. Fedor MJ, Uhlenbeck OC (1990) Substrate sequence effects on "hammerhead" RNA catalytic efficiency. Proc Natl Acad Sci USA 87:1668–1672
2. Khvorova A, Lescoute A, Westhof E et al (2003) Sequence elements outside the hammerhead ribozyme catalytic core enable intracellular activity. Nat Struct Biol 10:708–712
3. Martick M, Scott WG (2006) Tertiary contacts distant from the active site prime a ribozyme for catalysis. Cell 126:309–320
4. Pley HW, Flaherty KM, McKay DB (1994) Three-dimensional structure of a hammerhead ribozyme. Nature 372:68–74
5. Scott WG, Finch JT, Klug A (1995) The crystal structure of an all-RNA hammerhead ribozyme: a proposed mechanism for RNA catalytic cleavage. Cell 81:991–1002
6. Rupert PB, Ferre-D'Amare AR (2001) Crystal structure of a hairpin ribozyme-inhibitor complex with implications for catalysis. Nature 410:780–786
7. Lacroix-Labonte J, Girard N, Lemieux S et al (2012) Helix-length compensation studies reveal the adaptability of the VS ribozyme architecture. Nucleic Acids Res 40: 2284–2293
8. Masquida B, Beckert B, Jossinet F (2010) Exploring RNA structure by integrative molecular modelling. N Biotechnol 27:170–183
9. Helm M, Brule H, Giege R et al (1999) More mistakes by T7 RNA polymerase at the 5′ ends of *in vitro*-transcribed RNAs. RNA 5:618–621
10. Milligan JF, Uhlenbeck OC (1989) Synthesis of small RNAs using T7 RNA polymerase. Methods Enzymol 180:51–62
11. Solomatin S, Herschlag D (2009) Methods of site-specific labeling of RNA with fluorescent dyes. Methods Enzymol 469:47–68
12. Beckert B, Masquida B (2011) Synthesis of RNA by *in vitro* transcription. Methods Mol Biol 703:29–41
13. Price SR, Ito N, Oubridge C et al (1995) Crystallization of RNA-protein complexes I. Methods for the large-scale preparation of RNA suitable for crystallographic studies. J Mol Biol 249:398–408
14. Fechter P, Rudinger J, Giege R et al (1998) Ribozyme processed tRNA transcripts with

unfriendly internal promoter for T7 RNA polymerase: production and activity. FEBS Lett 436:99–103
15. Mörl M, Lizano E, Willkomm DK et al (2005) Production of RNAs with homogenous 5′ and 3′ ends. In: Hartmann RK, Bindereif A, Westhof E (eds) Handbook of RNA biochemistry, Wiley VCH Verlag Gmbh & Co, Weinheim, Germany, vol 1. pp 22–35
16. Ferré-D'Amaré AR, Doudna JA (1996) Use of *cis*- and *trans*-ribozymes to remove 5′ and 3′ heterogeneities from milligrams of *in vitro* transcribed RNA. Nucleic Acids Res 24:977–978
17. Nielsen H, Westhof E, Johansen S (2005) An mRNA is capped by a 2′, 5′ lariat catalyzed by a group I-like ribozyme. Science 309: 1584–1587
18. Hofacker IL, Fontana W, Stadler PF et al (1994) Fast folding and comparison of RNA secondary structures. Monatshefte für Chemie/Chemical Monthly 125:167–188
19. Canny MD, Jucker FM, Kellogg E et al (2004) Fast cleavage kinetics of a natural hammerhead ribozyme. J Am Chem Soc 126: 10848–10849
20. Ichetovkin IE, Abramochkin G, Shrader TE (1997) Substrate recognition by the leucyl/phenylalanyl-tRNA-protein transferase. Conservation within the enzyme family and localization to the trypsin-resistant domain. J Biol Chem 272:33009–33014
21. Milligan JF, Groebe DR, Witherell GW et al (1987) Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. Nucleic Acids Res 15:8783–8798
22. Jossinet F, Ludwig TE, Westhof E (2010) Assemble: an interactive graphical tool to analyze and build RNA architectures at the 2D and 3D levels. Bioinformatics 26:2057–2059

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