# Chapter 2

## Characterization of Hammerhead Ribozyme Reactions

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### Abstract

Hammerhead ribozymes are small catalytic RNA motifs ubiquitously present in a large variety of genomes. The reactions catalyzed by these motifs are both their self-scission and the reverse ligation reaction. Here, we describe methods for the generation of DNA templates for the subsequent in vitro transcription of hammerhead ribozymes. This is followed by a description of the preparation of suitable RNA molecules for both reaction types, and their kinetic analysis.

**Key words:** Hammerhead ribozyme, Catalytic RNA, Kinetic analysis, Cleavage reaction, Ligation reaction, Recursive PCR

### 1. Introduction

The hammerhead ribozyme belongs to the family of small endonucleolytic ribozymes (1). It consists of a catalytic core of conserved nucleotides that are flanked by three helical arms (Fig. 1), of which arms I and II engage in tertiary interactions. Only if these interactions take place can the catalytic activity of the hammerhead motif be observed under physiological conditions (2–5). The ribozyme catalyzes the establishment of an equilibrium of both self-cleavage and self-ligation and thus, the reaction is reversible (Fig. 1). The cleavage reaction produces two RNA fragments, of which the 3′ product features a 5′ hydroxyl group, while the 5′ product features a 2′, 3′ cyclic phosphate. Originally, the hammerhead motif was found in satellite RNAs (6, 7), circular single-stranded RNA molecules, which can accompany specific plant viruses, aggravating the virus' symptoms on the plant. These satellite RNA molecules replicate by a rolling circle mechanism (8), in which the hammerhead ribozyme cleaves and ligates in cis: it cuts multimeric linear forms into the monomers, which in turn could get their ends joined to

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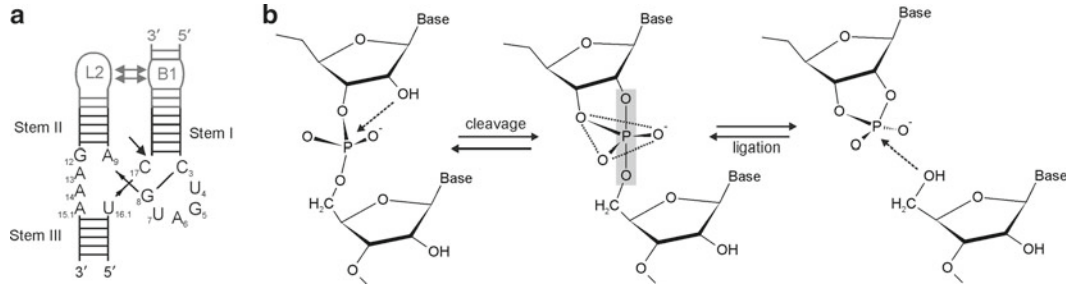


Fig. 1. The hammerhead ribozyme. (**a**) Shows the motif consisting of helical stems I, II, and III that surround the catalytic core with the indicated essential nucleotides. *Double-headed arrows* indicate tertiary interactions between elements in L2 and B1. The *arrow* shows the position of cleavage. Numbering is according to convention (9). (**b**) Shows the $S_N2$ mechanism, in which the 2′ hydroxyl attacks the adjacent 3′,5′ phosphodiester bond. In the transition state, a trigonal bipyramidal arrangement is reached (boxed in *grey*), that is resolved to yield the two products of the cleavage reaction. The 5′ product features a 2′, 3′ cyclic phosphate and the 3′ product a 5′ hydroxyl. These are also the substrates for the ligation reaction.

the circular monomeric form by the ribozyme motif. The latter reaction, however, has not been proven experimentally in vitro.

In recent years, the number of genomic positions, in which hammerhead ribozymes are found, has been dramatically extended (10, 11). After their discovery in various amphibians, schistosomes and cave crickets, hammerhead motifs were also found in a plant genome ((12), and references therein) or as a split version in various mammals (13). Most recently, we have shown the existence of this specific catalytic RNA as ultraconserved intronic motifs in all amniotes, including humans genome (14), as well as in a vast number of organisms, ranging from alpaca to zebrafish, indicating that the hammerhead ribozyme is ubiquitously present along the tree of life (15, 16). The biological function of these novel ribozyme motifs is presently under investigation in our laboratories, and a first, important step is the analysis of the cleavage and ligation reactions for each identified motif.

We provide here protocols for the generation of DNA templates, the preparation of the relevant RNA species, and the kinetic analysis of their reactions. The cleavage reaction of a conventional hammerhead ribozyme can be readily studied in cis, where it follows a first-order reaction kinetic. While the subviral plant pathogens may also circularize through a ligation reaction in cis, when they circularize, this is hard to reconstitute in vitro, as an access to molecules with both required ends, i.e., a 5′ hydroxyl group and a 2′, 3′ cyclic phosphate is limited. Because of this, the ligation reaction is best approximated in vitro by incubating the two reaction products of the cleavage reaction in a second-order reaction. While the in vitro ligation reaction thus proceeds in trans, its kinetic analysis is facilitated by using a large excess of one of the substrates. In this setup, the concentration of the latter substrate remains virtually unchanged, and the reaction can be analyzed according to

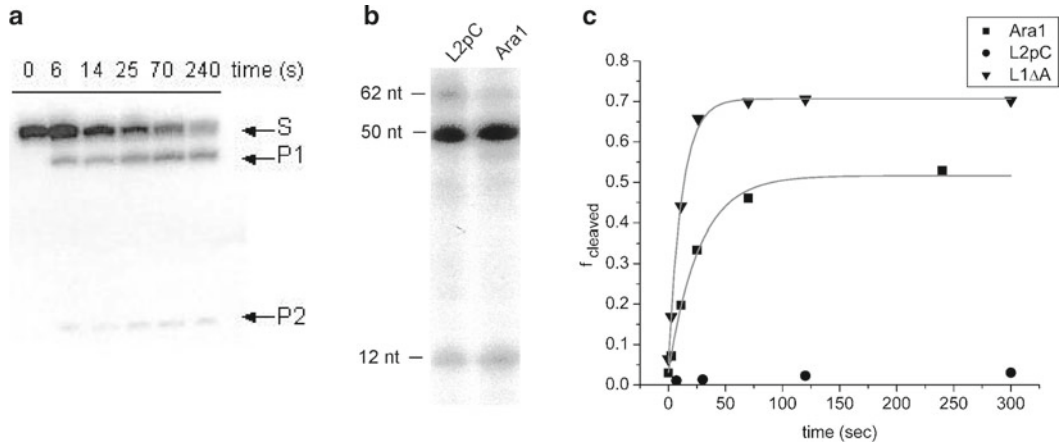


Fig. 2. Cleavage reactions of minimal and tertiary stabilized hammerhead ribozymes. (**a**) Shows a representative time course of a cleavage reaction of the tertiary stabilized *A. thaliana* hammerhead ribozyme Ara1. S, P1, and P2 denote substrate and products 1 and 2. (**b**) Complete cleavage of the 62 nucleotide (nt) long RNA in the two fragments of 50 and 12 nt is observed, at elevated $Mg^{2+}$ concentrations in the course of an in vitro transcription, both for Ara1 and the variant L2pC. This variant cannot form tertiary interactions, which can be inferred from (**c**), a comparison of the kinetic analysis of the cleavage reactions under physiological $Mg^{2+}$ concentrations. The cleaved fraction ($f_{cleaved}$) is plotted against time for the tertiary stabilized molecules Ara1 and L1ΔA, and the L2pC variant, which is inactive under these conditions. Reproduced from Ref. (12) with permission (www.plantcell.org; Copyright American Society of Plant Biologists).

pseudo-first order. In either case, it is essential that the reactions are performed in the presence of physiological concentrations of divalent metal ions like $Mg^{2+}$. Only then can the reaction of a true hammerhead ribozyme be monitored (Fig. 2), that relies on tertiary interactions between stems I and II (2, 3), which are also essential for the folding of this catalytic RNA (17).

## 2. Materials

All solutions should be prepared wearing gloves to avoid contamination with RNases, using deionized ultrapure water and analytical grade reagents (see Note 1).

### 2.1. Recursive PCR

1. Desalted DNA oligonucleotides are ordered from any suitable suppliers. Prepare stock solutions with a concentration of 100 μM. For the overlapping oligonucleotides they are diluted to working concentrations of 5 μM. Stock solutions of the *outer* primers are used undiluted.
2. For PCR, Taq polymerase is used together with the 10× buffer containing 10–20 mM $MgCl_2$, provided by the supplier.
3. The dNTP solution contains 2 mM of each dATP, dCTP, dGTP, and dTTP.

4. A PCR thermocycler.
5. A Stock of 10× TBE is prepared by dissolving 890 mmol Tris base and 890 mmol boric acid with 40 mL 0.5 M EDTA (pH 8.0) in 1 L $H_2O$. After autoclaving for 20 min, 100 mL are diluted 1:10 to yield 1 L 1× TBE.
6. Agarose.
7. Prepare a 10 mg/mL ethidium bromide solution.
8. DNA size standard with fragments in the range 50–200 bp, from the supplier of your choice.
9. The 6× DNA loading dye contains 10 mM Tris–HCl, pH 8.0, 60 mM $Na_2EDTA$, 60% (w/V) glycerol, 0.03% (w/V) bromophenol blue, and 0.03% (w/V) xylene cyanol.
10. If required, a gel extraction kit.
11. Any cloning vector lacking the T7 promoter sequence can be used to clone the PCR fragment of recursive PCR. We normally use the pJET1/blunt that Fermentas (now Thermo Fisher Scientific) has prepared upon request.
12. Competent *E. coli* cells, prepared using standard procedures or purchased commercially. LB medium and antibiotics depending on the resistance encoded in the used cloning vector.
13. For analytical DNA restriction, enzymes and their incubation buffers are required. Which enzyme to use depends on the cloning vector's features.
14. A gel documentation system with UV table suitable to record ethidium bromide stained gels.
15. DNA size marker with fragments in the kb range.
16. PCI mixture consisting of phenol: chloroform: iso-amyl alcohol (25:24:1; V:V:V). Work with PCI requires appropriate protective measures and should be performed under a fume hood.
17. A solution of 3 M NaOAc (pH 5.3) and ethanol, at concentrations of 100 and 70% (V/V).
18. A UV spectrophotometer.

### 2.2. PCR on Genomic DNA

1. PCR primers to amplify genomic ribozyme motifs.
2. Genomic DNA of the organism under investigation.
3. Material according to Subheading 2.1, steps 2–18.

### 2.3. In Vitro Transcription

1. Protective equipment to work with radio-labeled material.
2. Ingredients for the 10× transcription buffer are 400 mM Tris-HCl (pH 8.0), 200 mM $MgCl_2$, 20 mM spermidine, and 0.1% TritonX-100; ACG solution (5 mM of each ATP, CTP, and GTP), UTP solution (1 mM), RNase inhibitor (10 U/μL),

($\alpha$-$^{32}$P) UTP and T7 RNA Polymerase (20 U/μL), 0.5 M EDTA (pH 8.0).

3. An rNTPs mixture of 5 mM each.
4. A DNA oligonucleotide that is complementary to the core of the individual hammerhead motif.
5. Plastic syringes (1 mL).
6. Sterile glass wool.
7. Several 15-mL Falcon tubes and 1.5-mL reaction tubes with screw caps.
8. For gel filtration, 20 g of Sephadex G-50 fine are mixed in a bottle with 200 mL 1× TE containing 10 mM Tris–HCl and 1 mM EDTA (pH 8.0) and the mixture is boiled in a microwave for 30 s. Upon setting of the sephadex bed in the bottle, the buffer is poured off, and replaced by fresh buffer. Bring again to the boil and store after cooling at 4°C.

### 2.4. Gel Purification of RNA Species to Study Cleavage and Ligation Reactions

1. A vertical gel electrophoresis apparatus for polyacrylamide gels, with a power supply generating a voltage of 600 V.
2. Glass plates (25×20 cm), 0.5-mm thick spacers and comb.
3. A 12% polyacrylamide:bisacrylamide (19:1) solution with 7 M urea and 1× TBE.
4. Tetramethylethylendiamin (TEMED).
5. Ammoniumpersulfate (APS), dissolved in water to a concentration of 20% (w/V).
6. For the gel run 1× TBE.
7. A 10- or 20-mL syringe with injection needle.
8. Denaturing RNA loading buffer consisting of 95% formamide, 50 mM EDTA (pH 8.0), 0.03% (w/v) bromophenol blue, and 0.03% (w/v) xylene cyanol.
9. Heating block.
10. An RNA size marker.
11. Saran wrap.
12. A phosphorimager.
13. Sterile scalpels.
14. Gel extraction solution of 40% formamide (V/V), 0.7% (w/V) SDS in 1× TE.
15. A shaker for reaction tubes.
16. A solution of ethidium bromide with a concentration of 1 μg/mL 1× TBE.
17. Orbital shaker for trays.

### 2.5. Kinetic Analysis of the In Vitro Cleavage Reaction

1. Reaction buffer (10×) containing 100 mM Tris–HCl (pH 8.0), 100 mM NaCl and 1 mM EDTA (pH 8.0).
2. Start solution containing 6 mM $MgCl_2$ in 10 mM Tris–HCl (pH 8.0).
3. At least 10 reaction tubes with 10 µL denaturing RNA loading buffer (see Subheading 2.4, step 8), consecutively numbered and placed on ice.
4. Material for PAGE as described above (see Subheading 2.4, steps 1–12).
5. Computer software, like Prism to plot and fit the quantified kinetic reaction data.

### 2.6. Kinetic Analysis of the In Vitro Ligation Reactions

1. Reaction buffer (10×) containing 100 mM Tris–HCl (pH 8.0), 100 mM NaCl, and 1 mM EDTA (pH 8.0).
2. Start solution containing 25 mM $MgCl_2$ in 10 mM Tris–HCl (pH 8.0).
3. Material identical to Subheading 2.5, steps 3–5.

## 3. Methods

### 3.1. Recursive PCR

1. The DNA template contains the T7 RNA polymerase promoter sequence (TAATACGACTCACTATA), to which the sequence GGG, GCG, or GGC is added, depending on structural features of the resulting RNA (see Note 2). These triplets ensure high transcription yields (18). After either triplet, the sequence of the hammerhead ribozyme motif under investigation is added (Fig. 3). The resulting DNA template is therefore 20 nucleotides (nt) longer than the original ribozyme sequence.
2. The DNA template is then split up in an even number of DNA oligonucleotides that partially overlap (Fig. 4). It is crucial that all overlapping sequences for one DNA template have a similar predicted melting temperature (Tm) within 1°C. Tm values can be determined by a wide range of programs accessible via the internet (see Note 3). The size of the template determines the number of DNA oligonucleotides that are suitable to cover


Fig. 3. Design of the DNA template for in vitro transcription. In front of the DNA sequence encoding the hammerhead ribozyme, the sequence of the T7 promoter is added, followed by one of the indicated triplets.

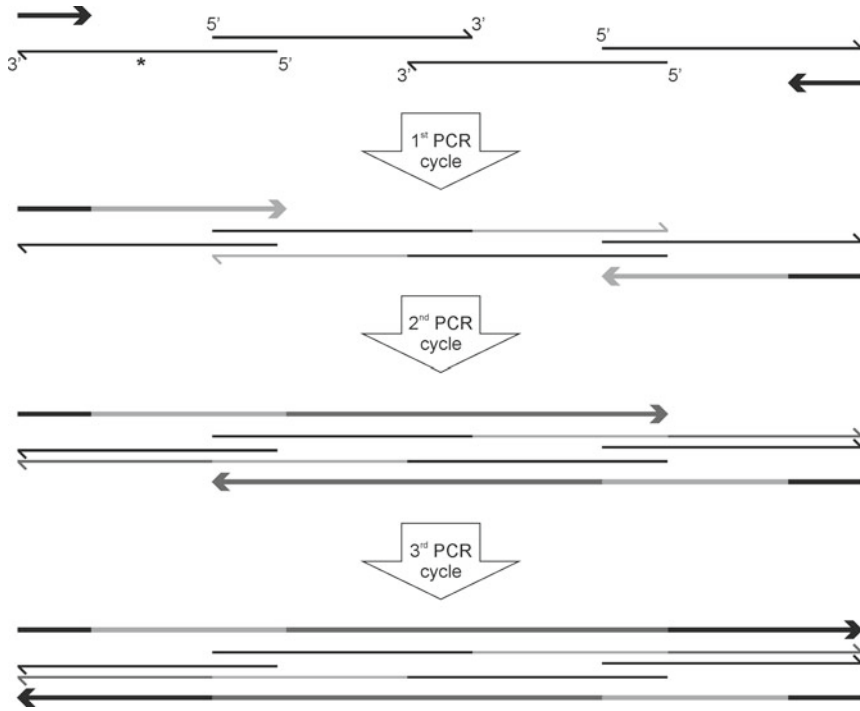


Fig. 4. Principle of recursive PCRs. An even number of overlapping DNA oligonucleotides (*thin arrows*) is designed in a way that the overlapping stretches have the same melting temperature (Tm). To these, two additional *outer* oligonucleotides with identical Tm to that of the overlapping stretches are added in excess (*thick black arrows*). In a first PCR round, the oligonucleotides are extended as indicated (*light grey lines*). In the second round, DNA strands get further extended (*dark grey lines*). In the third round and thereafter, only the *outer* oligonucleotides are extended to cover the entire sequence. The DNA oligonucleotide marked with an *asterisk* can be used in subsequent in vitro transcription reactions to prevent self-cleavage of the ribozyme.

the entire sequence. Currently, the efficient synthesis rate for oligonucleotides can easily yield 50–60 nt. On the basis of this and an average overlapping sequence of about 20 nt (depending on the sequence), the number of required DNA molecules ($N$) can be estimated according to (1) (see Note 4), in which $L$ is the length of the template:

$$L = 30\ \text{nt} * N + 20\ \text{nt} \quad (1)$$

Additionally to these DNA oligonucleotides, two *outer* primers are required that will amplify the template sequence eventually (Fig. 4). Their sequences are defined by the requirement to have a similar Tm value, as the overlapping sequences of the other DNA oligonucleotides have.

3. For PCR, a mixture is set up containing 1 μL of each overlapping DNA oligonucleotide (5 μM; see Note 5), 1 μL of each *outer* primer (100 μM), 2.5 μL 10× PCR buffer, 2.5 μL dNTP solution, and 0.5 μL Taq-Polymerase in a total reaction volume of 25 μL $H_2O$. This mixture is transferred to a PCR tube

and the PCR is started in a thermocycler with the following parameters:

| | | |
|---|---|---|
| First step | 1× | 30 s @ 95°C |
| Second step | 30× | 10 s @ 95°C<br>10 s @ $T_M$ of the used DNA oligonucleotides<br>10 s @ 72°C |
| Third step | 1× | 60 s @ 72°C |

4. Prepare a 1.5% standard agarose gel by dissolving 1.5 g agarose in 100 mL 1× TBE in a microwave oven. After the solution has cooled to about 60°C, add 5 µL ethidium bromide solution, cast the gel in a horizontal gel chamber, and insert a comb. After the gel has set, overlay with 1× TBE. Mix 10 µL of each PCR reaction with 2 µL loading dye and load next to a DNA standard covering the size range of the expected PCR product. Connect to a power supply and perform gel electrophoresis at a field strength of 10 V/cm.
5. Make sure to wear appropriate protective gear against the radiation, when analyzing the result by visualization on a UV table. Document the result by a gel documentation system. If no clear PCR product of the expected size is observed, the reaction can be repeated using a reduced amount of overlapping DNA oligonucleotides (see Note 5). As soon as a clear band is obtained use any cloning vector lacking the T7 promoter to clone the PCR product. Follow the manufacturer's instructions for the cloning vector to ligate the PCR fragment in the cloning vector. Transform in competent *E. coli* cells and on the next day, perform Plasmid mini-preparations according to the lab's standard protocols, or as described (19).
6. Use an analytical digest of the DNA of 5–10 prepared plasmids to identify clones with inserts of the expected size. Sequence 2–3 plasmids at any company that offers commercial sequencing. Upon identification of a clone with the correct DNA template (Fig. 3), prepare a 100 mL overnight culture of the original *E. coli* strain and prepare the plasmid DNA, according to your lab's standard protocols. This cell culture volume is expected to yield 100 µL solution of 1 µg/µL plasmid. Use a restriction enzyme that cleaves near after the last nucleotides encoding the ribozyme sequence (see Note 6) and linearize 10 µg of the plasmid preparation. To assess the completeness of the digest, analyze 1/100 (V/V) on a 1% agarose gel, using an equivalent amount of undigested plasmid for comparison and a conventional DNA size marker in the kb range. Proceed as above (step 4) and analyze the gel using a gel documentation system. Upon complete digest (see Note 7) purify the linear DNA by phenol extraction. For this purpose, mix equal amounts of the linear DNA solution and PCI in a 1.5-mL reaction tube,

vortex for 30 s and separate phases at room temperature in a table centrifuge at maximum speed for 5 min. Carefully remove the upper aqueous phase and transfer to a fresh 1.5-mL reaction tube. Add to the linear plasmid solution 1/10 (V/V) 3 M NaOAc, pH 5.3 and 3 (V/V) parts ethanol and incubate 20 min at –20°C. Place the tube in a cooling table centrifuge and start a run at maximum speed for 30 min at 4°C. Remove the supernatant, add 1 mL 70% ethanol and repeat centrifugation for 10 min. After removal of the liquid, dry the pellet by placing the open reaction tube in a heating block set to 50°C (see Note 8), and redissolve subsequently in 100 µL $H_2O$. Determine the solution's DNA concentration in a UV spectrometer.

### 3.2. PCR on Genomic DNA

1. If the ribozyme motif and its surrounding sequences in the genomic location under question are known, conventional forward and reverse PCR primers are designed. In front of the sequence of the forward primer, the sequence of the T7 RNA polymerase promoter and either triplet GGG, GCG, or GGC are added (Fig. 5), in analogy to the situation described in Subheading 3.1, step 1 (see Note 2). Perform a standard PCR on genomic DNA using the reaction components as indicated in Subheading 2.1, steps 2 and 3, using 5 µM primer solutions and at the PCR elongation temperature defined by the primers.
2. Analyze and clone the PCR product as described in Subheading 3.1, steps 4–6.

### 3.3. In Vitro Transcription

1. To generate the hammerhead ribozyme transcripts, linearized plasmid DNA serves as template that was generated either by recursive PCR (Subheading 3.1) or by PCR on genomic DNA (Subheading 3.2). Since the in vitro transcription is performed in the presence of ($\alpha$-$^{32}$P) UTP, appropriate protective measures against radioactivity must be in place in the laboratory and implemented for all work described in the subsequent sections. Mix by pipetting in a total reaction volume of 50 µL the

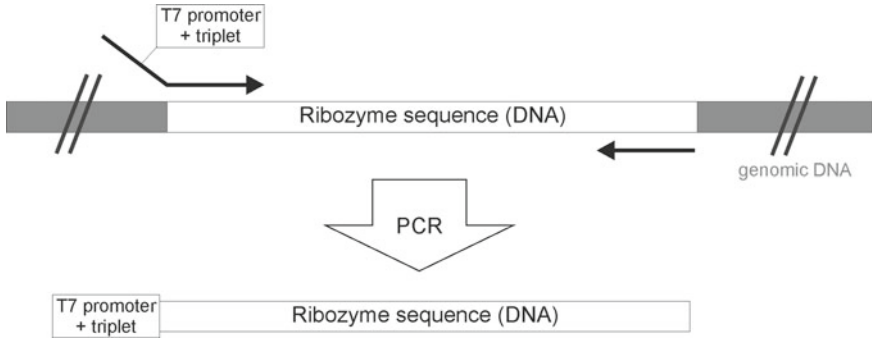


Fig. 5. Amplification of hammerhead ribozyme motifs from genomic DNA. To the forward PCR primer, the sequence of the T7 promoter and a transcription-promoting triplet (GGG; GGC or GCG) is added, yielding the indicated PCR product.

following ingredients: 200 ng linearized plasmid DNA, 5 µL 10× transcription buffer, 5 µL ACG solution, 5 µL UTP solution, 1 µL RNase inhibitor, 1 µL ($\alpha$-$^{32}$P) UTP, and start the reaction by the addition of 1 µL T7 RNA Polymerase. Incubate for 30 min at 37°C.

2. In a separate reaction, add a DNA oligonucleotide that is antisense to the core of the hammerhead ribozyme, at a concentration of 10 µM (see Note 9). This will disrupt the folding of the transcript in the hammerhead ribozyme structure and thus allows for preparation of full-length RNA for subsequent kinetic analysis.
3. For kinetic analysis of the in vitro ligation reaction, one of the cleavage products is required in a nonradio-labeled version. To generate this, perform a third in vitro transcription reaction as stated above (Subheading 3.3), however in the absence of ($\alpha$-$^{32}$P) UTP, and at a uniform rNTP concentration (see Note 10).
4. Stop all reactions after 30 min by the addition of 50 µL EDTA solution, 100 µL $H_2O$, and 200 µL PCI. Perform the phenol extraction as described above (Subheading 3.1, step 6).
5. After removing the upper aqueous phase, place it on a sephadex G-50 column (Fig. 6) to separate unincorporated nucleotides

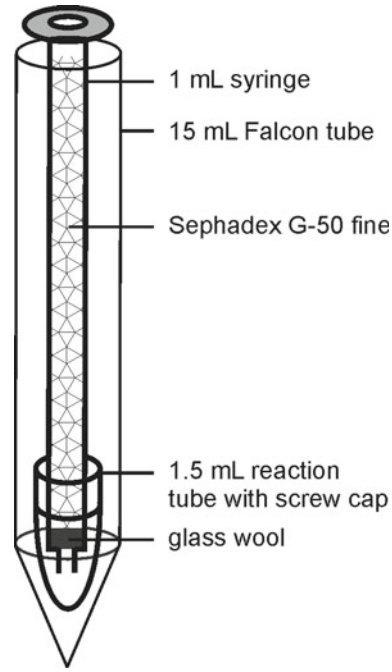


Fig. 6. Gel filtration column. A 1-mL plastic syringe is closed by pushing in sterile glass wool using the syringe's forcer. This serves as support for the gel bed. Place the syringe in an empty 15-mL Falcon tube. The syringe is then filled by pipetting Sephadex G-50 fine emulsion in the syringe. Let the material set by gravity flow. The column is set by centrifuging the syringe for 2 min at 1,000 × *g* within the Falcon tube. Discard the flow through, and place a 1.5-mL reaction tube (without the screw cap) in the 15-mL Falcon tube, into which the syringe is placed again, as shown here.

from the transcript by gel filtration. The sephadex column is prepared as described in the legend to Fig. 6. Add the aqueous phase after PCI treatment on top of the sephadex column and centrifuge the syringe within the 15-mL Falcon tube again for 2 min at 1,000×*g*. For the radio-labeled RNA, carefully discard the syringe in the radioactive waste and remove the 1.5-mL tube from the Falcon tube by using long forceps (see Note 11).

6. Add 1/10 volumes 3 M NaOAc (pH 5.3), and fill the reaction tube with 3 volumes ethanol. Precipitate RNA as described above (Subheading 3.1, step 6) and redissolve in 20 μL $H_2O$.

### 3.4. Gel Purification of RNA Species to Study Cleavage and Ligation Reactions

The transcription buffer contains $Mg^{2+}$ and this is sufficient to initiate the hammerhead ribozyme self-cleavage reaction, resulting in a mixture of the full-length RNA and the two cleavage products after transcription. The addition of an inhibitory DNA oligonucleotide in the transcription reaction (see Subheading 3.3) allows, however, to increase the fraction of uncleaved full-length RNA (see Note 9). For subsequent kinetic analysis, it is necessary to purify the full-length RNA to study the cleavage reaction, and the two cleavage products, to investigate the ligation reaction.

1. Carefully clean the glass plates of ca. 25×20 cm, and suitable spacers and comb, and set them up to cast a vertical gel.
2. Add 150 μL APS solution (see Note 12) and 60 μL TEMED to 60 mL polyacrylamide solution and cast the gel and immediately insert the comb.
3. After the gel has polymerized, remove the comb and flush the slots with 1× TBE, by using a syringe with an injection needle.
4. Place the glass plates with the gel in the electrophoresis chamber, fill the upper and lower tanks with 1× TBE, and connect to a power supply. Check the connections by pre-running the gel at a constant current of 40 mA.
5. Mix equal amounts (10–25 μL) of the RNA and the denaturing loading buffer and denature the RNA by placing the mixture on a heating block set to 95°C. After 2 min, snap cool by placing on ice.
6. Load the mixture in a freshly flushed gel pocket, next to an RNA size marker.
7. Separate the RNA species electrophoretically using a constant current of 40 mA. When the dyes of the loading buffer indicate good separation, after ca. 2 h, switch off the power supply. Carefully lift one gel plate.
8. For the radio-labeled transcripts, mix a small amount of (older) ($\alpha$-$^{32}$P) UTP, or any other β-emitter with the loading dye. Use this to draw with a micropipette an asymmetric pattern on those areas of the gel, where no RNA has been loaded.

Carefully cover the gel with saran wrap and expose to a phosphorimager screen for 10–30 min. Read the exposed screen and identify the bands of interest. Make a 1:1 print out of the gel area and place this under the glass plate with the gel and use the drawn pattern to overlay (see Note 13).

9. The gel with nonradio-labeled transcripts is displaced from both glass plates and incubated in a tray with ethidium bromide solution (see Note 14). After slow orbital shaking for 20 min, the gel is covered in saran wrap, and bands are visualized on the UV table of the gel documentation system.
10. Use a sterile scalpel to excise the bands of interest and move carefully to a fresh 1.5-mL reaction tube. Overlay the gel slice with gel extraction solution and place the closed tube in a shaker.
11. After shaking overnight, carefully pipette the liquid in a fresh 1.5-mL reaction tube. Perform a PCI treatment and precipitate RNA as described (see Subheading 3.1, step 6). Dissolve the RNA in 20–40 μL $H_2O$. Determine the concentration of the nonradio-labeled RNA spectrophotometrically.

### 3.5. Kinetic Analysis of the In Vitro Cleavage Reaction

1. Mix 10 μL of the radio-labeled full-length hammerhead ribozyme transcript with 80 μL $H_2O$ and 10 μL 10× reaction buffer.
2. Allow the RNA to refold by placing the tube for 1 min at 80°C, followed by snap cooling on ice.
3. Remove 10 μL to a reaction tube with denaturing RNA loading buffer as time 0 control.
4. Place the tube in a heating block set to 25°C and initiate the reaction by adding 10 μL start solution, resulting in an effective $Mg^{2+}$ concentration of 0.5 mM (see Note 15).
5. After suitable time points, e.g., 30 s, and 1, 2, 4, 6, 9, 12, 15 and 20 min, remove 10 μL aliquots from the reaction to prepared reaction tubes with denaturing RNA loading buffer (see Note 16).
6. Heat all 10 samples for 2 min at 95°C, snap cool on ice and load the entire content on a denaturing polyacrylamide gel. Perform PAGE to separate the reaction products (see Subheading 3.4).
7. After reading the phosphorimager plate, quantify the three relevant RNAs (FL: full-length; $P_1$: product 1 and $P_2$: product 2) using the phosphorimager's software.
8. Determine the fraction of cleaved RNA $f_{\text{cleaved}}$, according to (2):

$$f_{\text{cleaved}} = \frac{P_1 + P_2}{FL + P_1 + P_2} \tag{2}$$

9. Use a computer program to plot $f_{\text{cleaved}}$ against time. To obtain the end point of the cleavage reaction $f_{\infty,c}$ and first-order kinetic constant $k_{\text{cis}}$, fit the data according to (3), in which $f_0$ denotes the small fraction of transcripts that might have cleaved already before the addition of magnesium.

$$f_{\text{cleaved}} = f_0 + f_{\infty,c} * (1 - e^{-k*t}) \quad (3)$$

10. The constant $k_{\text{cis}}$ has the unit 1/s or 1/min. It allows together with the end point of the cleavage reaction $f_{\infty,c}$ to compare different hammerhead ribozymes.

### 3.6. Kinetic Analysis of the In Vitro Ligation Reaction

1. Mix 10 µL of the radio-labeled small product (S*) of the hammerhead ribozyme self-cleavage reaction with 30 µL $H_2O$ and 10 µL 10× reaction buffer.
2. Mix 10 µL of a 2 µM solution of the nonradio-labeled large product (L) of the hammerhead ribozyme self-cleavage reaction with 30 µL $H_2O$ and 10 µL 10× reaction buffer.
3. Allow the RNA solutions to refold separately by placing the tubes for 1 min at 80°C, followed by snap cooling on ice.
4. Mix the two solutions and remove 10 µL to a reaction tube with denaturing RNA loading buffer as time 0 control.
5. Place the tube in a heating block set to 25°C and initiate the reaction by adding 10 µL start solution, resulting in an effective $Mg^{2+}$ concentration of 2.5 mM (see Note 17).
6. After suitable time points, e.g., 1, 2, 4, 6, 9, 12, 15, 20, and 30 min, remove 10 µL aliquots from the reaction to prepared reaction tubes with denaturing RNA loading buffer.
7. Heat all 10 samples for 2 min at 95°C, snap cool on ice and load the entire content on a denaturing polyacrylamide. Perform PAGE to separate the reaction products (see Subheading 3.4).
8. After reading the phosphorimager plate, quantify the two relevant RNAs that are visible on the screen (FL: full-length; S*: small product) using the phosphorimager's software.
9. Determine the fraction of cleaved RNA $f_{\text{ligated}}$, according to (4)

$$f_{\text{ligated}} = \frac{\text{FL}}{\text{FL} + S^*} \quad (4)$$

10. Use a computer program to plot $f_{\text{ligated}}$ against time. To obtain the end point of the ligation reaction $f_{\infty,L}$ and pseudo-first-order kinetic constant $k_{\text{obs}}$, fit the data according to (5), in which $f_0$ denotes the small fraction of transcripts that might have ligated already before the addition of magnesium.

$$f_{\text{cleaved}} = f_0 + f_{\infty,L} * (1 - e^{-k*t}) \quad (5)$$

11. The constant $k_{cis}$ has the unit 1/min. It allows together with the end point of the ligation reaction $f_{\infty,L}$ to compare different hammerhead ribozymes.

## 4. Notes

1. We do not use Diethylpyrocarbonate (DEPC) that can be applied to alkylate and thus inactivate RNases, as we find it dispensable if normal lab standards are maintained.
2. The addition of the triplet might interfere with folding of the RNA into the hammerhead structure. It therefore is essential to compare the motif's predicted secondary structure with and without the triplet by using Mfold (20) or any other RNA folding program. In case that the presence of the optimal GGG changes the folding, GGC or GCG can be used alternatively (18).
3. Tm values determined with a given program are comparable with another, but not necessarily between programs. One example calculator is available at http://mbcf.dfci.harvard.edu/docs/oligocalc.html.
4. Since N has to be an even number, one can round the calculated value. For example, 100 nt is covered by $N = 8/3 = 2.66$, i.e., two DNA oligonucleotides; for a template sequence of 180 nt, the number comes down to $N = 16/3 = 5.33$, i.e., six DNA oligonucleotides will be required.
5. Since the overlapping DNA oligonucleotides are required only for the sake of providing the backbone from which the entire sequence is generated, one can dilute them manifold (1:5, 1:10, 1:20…). This is important when additional bands appear after PCR that have another size than the expected. Presumably these unwanted products are derived from primer dimers or the like. In case dilution of the overlapping DNA oligonucleotides does not yield a uniform PCR product of expected size, one needs to gel purify the desired PCR product using a gel purification kit. For that purpose, bands are visualized on a UV table, excised with a sterile scalpel, and processed according to the instructions provided by the manufacturer of the gel purification kit.
6. For linearization of plasmid DNA that is to be used for subsequent in vitro run off transcription, restriction enzymes producing blunt ends or 5′ overhangs are better suited than those

generating 3′ overhangs, as these might lead to extended RNA transcripts.

7. Occasionally, the digest remains incomplete. In that case it is advisable to gel purify the linearized plasmid, as described (see Note 5).
8. Alternatively, a speed vac can be used.
9. Frequently, the addition of an inhibitory oligonucleotide reduces the yield of transcript, including the full-length RNA dramatically, and additional shorter RNAs are observed. In this case, gel purification of the hammerhead ribozyme is required. In case the template has been generated by recursive PCR, the DNA oligonucleotide marked by a star (asterisk, Fig. 4) can be used. For templates from genomic DNA, a separate oligonucleotide has to be ordered.
10. In the generation of the cleavage products as substrates for ligation reactions do not include the inhibitory oligonucleotide (asterisk, Fig. 4), as in this case, a high fraction of self-cleaved material is desirable.
11. The success of the in vitro transcription of radio-labeled RNA can be immediately assessed by measuring the fraction of radionuclides in the column compared to the screw cap reaction tube, using a Geiger–Müller counter.
12. The APS solution must be stored at 4°C, and should be prepared freshly every month.
13. If no "old" radio source is available, take a small amount of the ($\alpha$-$^{32}$P) UTP.
14. The ethidium bromide solution might be reused if stored in a dark bottle.
15. It is essential to apply $Mg^{2+}$ concentrations in the low mM, or better sub-mM range. Only if the hammerhead ribozyme under investigation cleaves at these concentrations, it is a full, tertiary stabilized motif (2, 3, 12, 21). Depending on the organism, from which the ribozyme stems, the reactions might also be carried out at 37°C.
16. Depending on the idiosyncracies of the investigated motif, times have to be adjusted (22).
17. The preferred $Mg^{2+}$ concentrations used here is in the low mM range. This is attributable to the elevated RNA concentration that is present due to the pseudo-first-order reaction setup. Depending on the organism, from which the ribozyme stems, the reactions might also be carried out at 37°C.

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