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Design and Analysis of Hammerhead Ribozyme Activity Against an Artificial Gene Target

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Abstract

In vitro cleavage assays are routinely conducted to properly assess the catalytic activity of hammerhead ribozymes (HHR) against target RNA molecules like the dengue virus RNA genomes. These experiments are performed for initial assessment of HHR catalysis in a cell-free system and have been simplified by the substitution of agarose gel electrophoresis for SDS-PAGE. Substituting mobility assays enables the analysis of ribozymes in a more rapid fashion without radioisotopes. Here we describe the in vitro transcription of an HHR and corresponding target from T7-promoted plasmids into RNA molecules leading to the analysis of HHR activity against the RNA target by in vitro cleavage assays.

Keywords

Dengue; Flavivirus; Ribozyme; Catalysis; Electrophoresis; RNA; In vitro transcription

1 Introduction

Huge strides have been made in the development of antiviral strategies. One such approach involves the use of ribozymes, catalytic RNAs, with hammerhead ribozymes (HHRs) at the forefront [1–6]. There are a number of advantages to using HHR as a gene ablation tool. The catalytic activities of HHRs are independent of host cell's machinery [7]. Secondly, HHRs can simultaneously cleave a target RNA at more than one site by covalently linking several HHRs to form maxizymes [8].

HHRs cleave their target RNAs on the 3' end of the uracil located within an NUH nucleotide consensus (where N = any nucleotide, U = uracil, and H = any nucleotide but G) in a magnesium ion-dependent manner, provided there are sequence homologies between the ribozyme arms and the corresponding target sequence [9] (Fig. 1).

Initial assessment of HHR activity is most effectively executed through in vitro cleavage assays. The in vitro cleavage reactions we have used are adopted from a protocol developed by Shao et al. [10]. These assays are performed in two general steps: (1) In vitro transcription of HHR and the corresponding artificial target, each from a separate T7

promoter and (2) a general in vitro cleavage reaction to assess the ability of these ribozymes to catalyze the cleavage of the intended substrates.

The success of in vitro cleavage assays depends on the precise determination of the experimental conditions necessary for optimal HHR activity. Successful determination of the optimal in vitro cleavage conditions is achieved by varying the amounts of HHRs used, the concentration of MgCl_2 necessary for HHR catalysis, and the time necessary to achieve optimal catalysis. We previously produced T7-promoted anti-dengue virus (DENV) HHRs and corresponding target DENV regions and showed through in vitro cleavage analysis that several of our HHRs were capable of catalyzing the cleavage of DENV RNA substrates [1]. The protocol presented here can be tailored for use with any HHR/target RNA pair.

2 Materials

2.1 In Vitro Analysis of HHR Cleavage Activity

The success of in vitro transcription and in vitro cleavage assays is greatly increased if performed under RNase-free conditions. To ensure this, wipe down all gel chambers and pipettors with an RNase-eliminating solvent. Sterilize all glassware and solutions prior to use unless indicated otherwise. All water used in making the described buffers should be certified RNase free by the manufacturer or at least millipure, diethyl pyrocarbonate (DEPC) treated, and autoclaved prior to use. Store all reagents at room temperature unless indicated otherwise. Closely follow all waste disposal regulations when disposing of waste materials. All concentrations given in parentheses are the final concentrations.

1. 1 M Tris-HCl (pH 8.0): Mix 121.1 g of Tris base with 700 mL of millipure H_2O by stirring. Add 42 mL concentrated HCl to reach pH of 8.0. Fine adjust to the desired pH (8.0) with concentrated HCl if needed (*see Note 1*). Add millipure H_2O until final volume is 1 L and autoclave.
2. 5 M NaCl stock: Weigh 292.2 g NaCl and add to 800 mL millipure water. Mix until dissolved using a stir bar and stir plate, add millipure H_2O to 1 L, and autoclave.
3. 1 M NaCl: In an autoclaved graduated cylinder place 10 mL 5 M NaCl with 40 mL DEPC-treated H_2O . Mix well, and place in an autoclaved bottle.
4. 1.5 M MgCl_2 stock solution: Weigh 304.95 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and add to 800 mL millipure water. Mix until dissolved using a stir bar and stir plate. Add millipure H_2O to 1 L and autoclave.
5. RNase inhibitor: “RNaseout” purchased from Life Technologies. Only 40 U (1 μL) per reaction is needed.
6. HHR: The T7-promoted forward and antisense forms should be obtained from a gene or a primer synthesis company as DNA, annealed at 95 °C for 5 min,

¹Many electronic pH meters are unable to accurately determine the pH of concentrated Tris solutions. Be sure to use an appropriate probe. pH is temperature dependent: ~0.03 pH units per 1 °C increase. Make sure that the Tris solution is at room temperature before making final pH adjustments.

immediately cooled on ice, and then in vitro transcribed from the T7 promoter (*see* Subheading 3.2) prior to use in the described in vitro cleavage reactions.

7. Artificial target: This is a mimic of your gene or gene segment of choice and is in vitro transcribed from a T7 promoter (*see* Subheading 2.1, **item 1**).
8. In vitro transcription kit: We use the *Megascriptshort* and *Megascript* kits from Life Technologies (*see* Note 2).
9. Agarose gel extraction kit: We use the *Promega SV Gel* and *PCR Clean-Up System*.
10. GuTC extraction buffer: In lieu of GuTC buffer, “Trizol,” from Life Technologies can be used. Be sure to add the prescribed amount of LiCl to the GuTC extraction buffer.
11. RNA gel loading buffer: 95 mL formamide, 18 mM EDTA (pH 7.5), 0.025 % SDS, 0.05 g bromophenol blue, and 0.05 g xylene cyanol. Add enough DEPC-treated water to make the total volume 100 mL and then autoclave after mixing.
12. RNase-free ethidium bromide (10 mg/mL): Dissolve 1 g of ethidium bromide with 100 mL DEPC-treated dH₂O. Mix by stirring, and store in the dark at 4 °C.
13. 10× MOPS running buffer: Add 41.85 g 4-morpholinopropanesulfonic acid (MOPS-free acid) and 6.80 g sodium acetate–3H₂O to 800 mL DEPC-treated dH₂O and stir until completely dissolved. Add 20 mL of a DEPC-treated 0.5 M Na₂EDTA solution and adjust pH to 7.0 with 10 M sodium hydroxide or glacial acetic acid. Adjust volume to 1 L with DEPC-treated dH₂O (*see* Note 3).

3 Methods

3.1 Selection of HHR Target Sites on DENV-2 Genome

Align all sequences of the gene of interest using ClustalX [11, 12]. Select HHR target sites by scanning for conserved sequences that contain the nucleic acid triplets GTC, GTA, ATC, or CTC, since HHR-mediated cleavage occurs more efficiently when these triplets are targeted [28]. At the minimum a target sequence containing an NUH triplet (*see* Subheading 1) is required. For example, our primary criterion for the selection of a DENV target site was that this target site must be present in all 29 strains of DENV-2. Another important criterion for selecting a suitable site for HHR cleavage is the length of conserved flanking arms which determine the level of specificity of the ribozyme. The length of each arm should range from 5 to 10 bp [1]. Once target sites are selected, design the ribozyme arms such that the 5′ arm of the HHR will be a complementary base pair to the 3′ end of the target site flanking the NUH triplet and the 3′ arm of the HHR will be a complementary base pair

²*MEGAscriptshort* in vitro transcription kits are used to produce RNA transcripts less than 500 nucleotides in length, such as HHRs which are approximately 40 nucleotides in length. *MEGAscript* kits are typically used to produce RNA transcripts that are larger than 500 bases, such as the DENV-2 target sequence we produced [1].

³Always protect MOPS from light by wrapping container in aluminum foil, and store 10× MOPS at 4 °C. If the solution turns yellow, make anew.

to the 5' end of the target site flanking the NUH triplet. Ensure that the lengths of the binding arms are not longer than the targeted conserved region.

3.2 In Vitro Transcription

3.2.1 Linearization of the Ribozyme Plasmid and DENV Template

1. In separate reaction tubes place 2.5 µg ribozyme and 1.5 µg DENV target with 1 µL of the restriction endonuclease (REN) of interest (*see* Note 4 and Fig. 2).
2. Allow these REN reactions to proceed for 4 h at a temperature that is optimal for REN activity to ensure that all plasmids in the reaction tube are linearized (*see* Note 5).
3. Treat the linearized plasmids with 1 µL Antarctic phosphatase (New England Biolabs) for 15 min at 37 °C to prevent self- ligation of the digested ends.
4. Heat inactivate the Antarctic phosphatase and REN by incubating the reaction tubes at 65 °C for 20 min. This will prevent self-ligation of the digested plasmid.
5. Separate bands by 1 % agarose gel electrophoresis, excise the band of interest using a scalpel, and gel purify this fragment using any agarose gel extraction kit.

3.2.2 In Vitro Transcription—In vitro transcribe the HHR and target using the *Megashortscript* (for HHR RNA production) and *Megascript* (for target RNA production) kits that each contains the T7 polymerase (Life Technologies, USA). Below is an in vitro transcription kit protocol as we use it in the lab.

1. Immediately following gel extraction, place 1 µg of the ribozyme and target in separate 1.5 mL Eppendorf tubes.
2. To each tube add 2 µL of each triphosphated nucleotide, 2 µL reaction buffer, and 2 µL enzyme mix (*see* Note 6). Add nuclease-free or DEPC-treated water.
3. Incubate the in vitro transcription reactions containing the target plasmid for 4 h at 37 °C. The in vitro transcription reactions containing the ribozyme require a longer incubation of 16 h at 37 °C (*see* Note 7). DNase digestion is not necessary since the quantity of digested plasmid is in a much lower concentration relative to RNA, but DNase treatment can be performed if desired.

⁴We insert the vesicular stomatitis virus transcription termination signal (5'-TTTTTTCATA-3'; [13]) immediately downstream of the gene or the gene segment we wish to transcribe but immediately upstream of the restriction site used to cleave the vector prior to in vitro transcription. Be sure to insert this sequence for efficient transcription of your HHR target.

⁵If digestion of the parental DNA vector prior to in vitro transcription is not performed circular DNA plasmid templates will generate extremely long, heterogeneous RNA transcripts. To insure proper digestion, linearized DNA templates should be examined on an agarose gel to confirm complete digestion. Although any restriction enzyme may be used, one should avoid restriction enzymes that leave 3' overhanging ends as this can lead to low-level transcription [14]. Be sure to choose a restriction site that is digested by an REN that can be inactivated by heat. Most RENs can be completely inactivated at 65 °C for 20 min.

⁶Once thawed, store the ribonucleotides on ice while assembling the transcription reaction. The RNA polymerase enzyme mix should be placed on ice immediately following removal from the freezer since it is stored in glycerol and will not freeze at -20 °C. Keep the 10× reaction buffer at room temperature while assembling the reaction. Mix these components in a single master mix, large enough to accommodate all samples to be transcribed and pipette from that vessel into the sample tubes containing the linearized plasmid. This will decrease your chances for RNase contamination.

⁷For transcripts shorter than 500 nt, a longer incubation time of up to 16 h is advantageous since more transcription initiation events are required to synthesize a given mass amount of RNA compared to transcription of longer transcripts.

3.2.3 RNA Isolation

1. To each sample tube add DEPC-treated water for a final volume of 100 μ L.
2. Add an equal volume of GuTC extraction buffer, mix very well, and incubate the samples at room temperature for 5 min.
3. Add 100 μ L of phenol, mix very well, and incubate at room temperature for 5 min. If you have chosen to use Trizol for this step, the addition of phenol is not necessary since it is included.
4. Add 100 μ L of chloroform–isoamyl alcohol (*see* Note 8), close cap, and mix manually by vigorously shaking tubes to create an emulsion. Do not vortex. Spin at 12,000 rpm in a table microcentrifuge, at 4 °C, for 15 min (*see* Note 9). Transfer the top aqueous to a fresh, prechilled 2 mL microcentrifuge tube. The top aqueous phase contains all the isolated RNA molecules.
5. Add 100 μ L chloroform–isoamyl alcohol (24:1) to the transferred aqueous phase. Close cap and shake vigorously to create an emulsion. Spin at 12,000 rpm in a table microcentrifuge, at 4 °C, for 15 min. Once again transfer the top aqueous phase to a fresh, prechilled 2 mL microcentrifuge tube (*see* Note 10).
6. Add 2 volumes of 100 % ethanol and 0.2 μ g of glycogen to each sample, and incubate samples overnight at –20 °C (*see* Note 11).
7. The following day spin samples at 12,000 rpm in a table microcentrifuge, at 4 °C, for 30 min. Remove as much liquid as possible without disturbing the pellet. Air-dry the RNA pellet at room temperature ensuring that all liquid has dissipated (*see* Note 12).
8. Resuspend the RNA pellet in a suitable amount of DEPC-treated water or nuclease-free water from your favorite manufacturer. Determine the concentration spectrophotometrically.

3.3 Ribozyme In Vitro Cleavage Reaction

1. Design separate experiments to vary (a) the amount of in vitro-transcribed ribozymes from 0 to 50 pM; (b) the concentration of $MgCl_2$ from 5 to 50 mM (from a 1 M stock), in a solution containing 50 mM Tris–HCl, pH 8.0 and 40 U (1 μ L) of RNase inhibitor; and (c) the incubation time from 0 to 30 min in 5-min increments. The total volume of these reactions should be no more than 10 μ L.

⁸Take care when using chloroform as overexposure can result in skin and eye irritation, mutagenic for mammalian somatic cells, and may be toxic to kidneys, liver, and heart. Repeated or prolonged exposure to the substance can produce damage to target organs.

⁹In our experience spinning RNA at a maximum of 12,000 rpm greatly increases yield since centrifugation at speeds greater than this can lead to the shearing of RNA transcripts.

¹⁰Keep samples on ice or at 4 °C as much as possible. This will enhance RNA yield.

¹¹Overnight incubation of RNA precipitations at –20 °C greatly facilitates precipitation of short (>500 nt) transcripts. LiCl is generally the precipitant of choice for RNA. However this method is contraindicated for RNA molecules smaller than 300 nucleotides.

¹²Do not wash pellet in 70 % EtOH since glycogen is soluble in water and even the water contained in the 70 % EtOH can prematurely solubilize the pellet. Guard against overdrying the RNA pellet as this will make resuspension very difficult.

2. Place 10 pM of in vitro-transcribed target and the corresponding in vitro-transcribed HHR (0–50 pM) in the same 0.5 mL thin-walled PCR tube containing 50 mM Tris–HCl, pH 8.0.
3. Heat denature the components by placing the PCR tubes in the thermocycler at 85 °C for 3 min, and immediately place on ice (*see Note 12*).
4. Initiate the cleavage reaction by adding 0.1 µL of 1 M MgCl₂ (final concentration of 10 mM) and 1 µL (40 U) of RNase inhibitors. Mix by gently pipetting samples, and incubate at 37 °C for 0–30 min.
5. Stop the HHR cleavage reaction by adding 30 µL (i.e., 3×volume) of the gel loading buffer.

3.4 2 % Agarose/ Formaldehyde/MOPS Gel Electrophoresis

1. Rinse the gel box, gel combs, and gel caster you will use for RNA electrophoresis with copious amounts of millipore water, making sure to remove any salt deposits from prior electrophoresis experiments. Air-dry components: a 50 °C incubator can be used to facilitate the drying process. Wipe components with an RNase-eliminating solvent. Overnight soaking of the gel caster and comb in the RNase-eliminating solvent is recommended but not essential.
2. In a 250 mL Erlenmeyer flask melt 2 g agarose in 70 mL RNase-free ddH₂O and cool down to 60 °C.
3. In a fume hood, add 20 mL 37 % formaldehyde and 10 mL 10× MOPS (*see Note 14*).
4. Cast the gel in the fume hood. Place gel comb in gel caster and place gel comb/caster assembly in gel box. Pour the melted 2 % agarose/formaldehyde/MOPS into the gel caster, cover, and allow the gel to cool and polymerize.
5. Heat the in vitro cleavage samples to 65–70 °C for 5 min, and load 30 µL into each well of the cooled/polymerized 2 % agarose/ formaldehyde/MOPS gel (*see Note 15*).
6. Run the gel in 1× MOPS buffer for 1 h at 105 V (*see Note 16*).
7. Rinse the gel with millipore water and place in a receptacle suitable for staining and destaining. We have found that used pipette tip boxes are ideal for this. Stain the gel for 1 h in DEPC-water containing 10 µg/mL ethidium bromide (*see Note 17*). Destain the gel in millipore water for a minimum of 5 min. Change the water and destain for another 10 min (*see Note 18*). Photograph the gel or destain more if necessary. It may be also necessary to resolve the bands more. *See Nawtaisong et*

¹⁴Always use a fume hood when pouring or decanting formaldehyde. EB is a potential carcinogen. Always wear appropriate protection when handling formaldehyde or EB.

¹⁵Heating samples at this stage in the presence of formide will denature RNA [15], enabling their migration on a MOPS gel while providing protection from RNases [16].

¹⁶It is best to recirculate the running buffer. If recirculation is not available, stop the gel after 30 min and manually mix the buffer by pipetting from one side to the other.

al. [1] for an example of a finished gel depicting the HHR cleavage product resulting from an in vitro cleavage assay.

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¹⁷The time given is just a baseline. The amount of time necessary to stain gels with ethidium bromide depends on the amount of RNA in the gel, the concentration of the gel itself, and the desired concentration of EB used. Formaldehyde gels are notoriously difficult to stain with ethidium bromide, since the dye appears to bind to the gel matrix and cause high backgrounds. The background can be minimized by limiting the exposure to the staining solution to less than 5 min and destaining in water. Alternatively, the gel can be soaked in water for 10–15 min prior to staining to remove the formaldehyde and allow the RNA to renature, thus increasing its ability to bind ethidium bromide.

¹⁸The time given is just a baseline. The amount of time necessary to stain gels with ethidium bromide depends on the amount of RNA in the gel, the concentration of the gel itself, and the desired concentration of EB used.

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Fig. 1.

Design and construction of a typical hammerhead ribozyme (HHR). HHRs are small ribonucleic-based RNAs that cleave target RNA in a sequence-specific manner. Catalysis is initiated by base pairing of the 5' helix I and 3' helix III arms of the HHR to complementary 3' and 5' bases on the target RNA to form helices I and III, respectively. The catalytic core of the HHR, helix II, is responsible for cleavage at a 5'-NUH-3' triplet site on the target RNA, where N can be any 4 nucleotide and H can be A, C, or U. Target RNA is shown in purple with "Y" depicting each nucleotide residue to be bound by the HHR. The HHR is in black with "X" depicting each nucleotide residue of the 5' and 3' arms

Fig. 2.

(a) Diagram of a typical T7-promoted ribozyme construct, T7-HHR. We typically insert T7-HHR into a Bluescript expression vector (Invitrogen) prior to in vitro transcription. (b) Schematic representation of the predicted in vitro-produced transcripts (T1 and T2) from a T7-promoted hypothetical gene (Gene X) following in vitro transcription and cleavage products resulting from HHR activation (C1, C2, and C3). T7 term = T7 termination sequence. HHR cleavage site is indicated by *arrows*