Chapter 2

Selective RNase H Cleavage of Target RNAs from a tRNA Scaffold

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Abstract

In vivo overproduction of tRNA chimeras yields an RNA insert within a tRNA scaffold. For some applications, it may be necessary to discard the scaffold. Here we present a protocol for selective cleavage of the RNA of interest from the tRNA scaffold, using RNase H and two DNA oligonucleotides. After cleavage, we show that the RNA of interest can be isolated in a one-step purification. This method has, in particular, applications in structural investigations of RNA.

Key words: Recombinant RNA, tRNA scaffold, RNase H cleavage, RNA purification

1. Introduction

Our protocol of in vivo overproduction of tRNA chimeras yields an RNA insert within a tRNA scaffold (1). This system should have broad application in structural, biochemical, and biophysical investigations of RNA (1, 2). For some applications, it may be necessary to remove the scaffold from the target RNA. We considered several strategies: using either a dedicated ribozyme (3) or DNAzyme (4) or cleaving with RNase H using a pair of guide DNA oligonucleotides. Dual cis-acting ribozyme constructs have been used for in vitro transcribed RNA, but this approach is not compatible with the tRNA scaffold topology (5). In our hands, the method that worked best is RNase H cleavage and thus we have developed a technique for selectively cleaving the RNA of interest from the tRNA scaffold, using RNase H and two DNA oligonucleotides.

RNase H specifically cleaves the RNA strand of DNA–RNA heteroduplexes (6, 7). The strategy is therefore to use two "guide" DNA oligonucleotides complementary to the 5′ and 3′ halves of

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tRNA moiety of the chimera. Hence, upon incubation with RNase H, the scaffold is hydrolyzed, whereas the RNA of interest is left intact. We found that 60 pmol of *Escherichia coli* RNase H is usually sufficient to cleave 1 nmol of RNA in 2 h at 30°C (8). Commercially available RNase H can be used; however, for large-scale applications, using RNase H purified in-house is more cost-effective. The most critical step is the hybridization of the RNA with the DNA oligonucleotides. Here, as an example of the approach, we present the cleavage and the purification of the decoding site of the 16S ribosomal RNA (9). We propose a simple method to estimate the length of the RNA product by SDS-PAGE followed by a *Stains-all* staining (10).

2. Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 15 MΩ cm at 25°C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1. Production and Purification of RNase H

1. pET28a-(6×His)RNase H fusion protein expression vector (see Note 1).
2. Competent BL21(DE3) *E. coli* cells (Novagen).
3. Kanamycin: Prepare a stock solution of 37 mg/mL of kanamycin sulfate salt in H_2O and filter-sterilize. Store at 4°C.
4. Luria Broth (LB medium).
5. LB agar plates: Add 12 g bacto agar to 1 L LB medium before autoclaving. To prepare plates, allow medium to cool until flask or bottle can be held in hands without burning, then add 1 mL of the kanamycin stock, mix by gentle swirling, and pour or pipette approximately 30 mL into each sterile Petri dish (100 mm diameter). The final concentration of ampicillin should be 37 μg/L.
6. Isopropyl β-D-1-thiogalactopyranoside (IPTG).
7. 10% Sodium dodecyl sulfate (SDS).
8. 40% Acrylamide/bis-acrylamide solution (37:1).
9. 10% Ammonium persulfate.
10. *N,N,N,N*′-tetramethyl-ethylenediamine (TEMED).
11. SDS-PAGE running buffer: 0.025 M Tris–HCl (pH 8.3), 0.192 M glycine, and 0.1% SDS.
12. SDS loading buffer (5×): 0.3 M Tris–HCl (pH 6.8), 10% (w/v) SDS, 25% (v/v) β-mercaptoethanol, 0.1% (v/v) bromophenol blue (BPB) solution, and 45% (v/v) glycerol. Prepare 20 mL

of loading buffer, leave 1 mL at 4°C for current use, and store the remaining aliquots at −20°C.

13. BPB solution: Dissolve 0.1 g BPB in 100 mL water.
14. 1 M Tris–HCl (pH 8).
15. 1 M Tris–HCl (pH 7.5).
16. Imidazole.
17. Resuspension buffer: 50 mM Tris–HCl buffer (pH 7.5) and 100 mM NaCl.
18. Elution buffer: 50 mM Tris–HCl buffer (pH 7.5), 100 mM sodium chloride, and 500 mM imidazole.
19. Storage buffer: 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, and 50% glycerol.
20. Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8. Add about 100 mL water to a 1-L graduated cylinder or a glass beaker. Weigh 181.7 g Tris base and transfer to the cylinder. Add water to a volume of 900 mL. Mix and adjust pH with HCl. Make up to 1 L with water. Store at 4°C.
21. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8. Weigh 60.6 g Tris base and prepare a 1 L solution as in previous step. Store at 4°C.
22. Polypropylene conical tubes, 50 mL.
23. Baffle-bottomed shake flask.
24. Temperature-controlled shaking incubator.
25. Mechanical device to disrupt *E. coli* cells (e.g., a sonicator, French press, or cell homogenizer).
26. Apparatus for gel electrophoresis.
27. Ni-NTA superflow resin (Qiagen): Pack 20 mL of the Ni-NTA superflow resin in an XK 16/40 column (GE Healthcare), following the supplier's instructions.
28. ÄKTA FPLC chromatography system (or equivalent).

2.2. RNase H Cleavage

1. Purified RNA-tRNA chimera (see Chapter 1).
2. DNA oligonucleotides (see Note 2).
3. Purified *E. coli* Ribonuclease H (from Subheading 3.1 of this protocol).
4. Hot plate.
5. ÄKTA FPLC chromatography system (or equivalent).
6. Mono-Q HR 10/10 (GE healthcare).
7. RNase-free water.
8. 1 M NaCl.
9. 1 M Tris–HCl (pH 7.4).

10. 1 M $MgCl_2$.
11. TBE Buffer (5×): 54 g Tris base, 275 g boric acid, and 4.7 g EDTA per liter of water. TBE buffer is typically stored indefinitely at room temperature.
12. Urea–acrylamide gel running buffer (0.5× TBE): Mix 100 mL of 5× TBE and 900 mL of water.
13. 5× Loading Buffer: Mix 50% (v/v) glycerol and 0.2% (w/v) xylene cyanol.
14. 40% Acrylamide/bis-acrylamide solution (19:1).
15. 1 M Potassium phosphate buffer, pH 7.0: Weigh out 107.12 g of K_2HPO_4 and 52.39 g of KH_2PO_4. Transfer to a 1-L graduated cylinder, add water to a volume of 1 L, and mix to dissolve.
16. 8 M Urea: Weigh out 480.48 g of urea. Transfer to a 1-L graduated cylinder, add water to a volume of 1 L, and mix to dissolve (see Note 3).
17. Buffer A: Weigh out 240.24 g of urea. Transfer to a 1-L graduated cylinder, add 40 mL of potassium phosphate buffer pH 7.0, add water to a volume of 1 L, and mix to dissolve.
18. Buffer B: Weigh out 240.24 g of urea and 58.44 g of NaCl. Transfer urea and NaCl to a 1-L graduated cylinder, add 40 mL of potassium phosphate buffer pH 7, add water to a volume of 1 L, and mix to dissolve.
19. Apparatus for gel electrophoresis.
20. Handheld UV lamp.

2.3. Stains-All *Staining*

1. DNA oligonucleotides (see Note 4).
2. 40% Acrylamide/bis-acrylamide solution (37:1).
3. 10% Ammonium persulfate solution.
4. *N,N,N,N'*-TEMED.
5. SDS-PAGE running buffer: 0.025 M Tris–HCl (pH 8.3), 0.192 M glycine, and 0.1% (w/v) SDS.
6. SDS loading buffer (5×): 0.3 M Tris–HCl (pH 6.8), 10% (v/v) SDS, 25% β-mercaptoethanol, 0.1% (v/v) BPB solution, and 45% (v/v) glycerol. Prepare 20 mL and leave 1 mL at 4°C for current use and store the remaining aliquots at –20°C.
7. BPB solution: Dissolve 0.1 g BPB in 100 mL water.
8. Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8. Add about 100 mL water to a 1-L graduated cylinder or a glass beaker. Weigh out 181.7 g Tris base and add water to a volume of 900 mL. Mix and adjust pH with HCl. Make up to 1 L with water. Store at 4°C.
9. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8. Weigh out 60.6 g Tris base and prepare a 1 L solution as in previous step. Store at 4°C.

10. Apparatus for gel electrophoresis of protein.
11. Rinsing solution: 25% (v/v) isopropanol.
12. *Stains-all* (Sigma).
13. *Stains-all* staining buffer: Mix 30 mL of Tris–HCl (pH 8.8), 75 mL formamide, and 250 mL isopropanol, followed by addition of 0.025% (w/v) *Stains-all*. Add water to a volume of 1 L (see Note 5).
14. Lighttight containers.
15. Orbital shaker.

3. Methods

3.1. Production and Purification of RNase H

1. Transform 50 μL of competent BL21(DE3) cells with 10–100 ng of pET28a-(6×His)RNase H fusion protein expression vector, and spread 5–200 μL on an agar plate containing 37 μg/mL kanamycin. Incubate the plate overnight at 37°C.
2. Inoculate 100 mL LB medium in a 500-mL baffle-bottomed shake flask with a single colony from the transformation. Shake overnight at 220 rpm and 37°C.
3. Add 25 mL of the saturated overnight culture to 1 L fresh LB medium containing 100 μg/mL kanamycin in a baffle-bottomed shake flask.
4. Shake the flasks at 250 rpm and 37°C until the cells reach mid-log phase, OD_{600} of approximately 0.6.
5. Add IPTG to a final concentration of 1 mM. Continue shaking for 3–4 h.
6. Recover the cells by centrifugation for 15 min at 6,000×*g* and 4°C (see Note 6).
7. Resuspend the cells in resuspension buffer, using at least 10 mL per gram of wet cell pellet.
8. Lyse the cell suspension using a mechanical device to disrupt the *E. coli* cells (e.g., a sonicator, French press, or cell homogenizer) and centrifuge the disrupted cell suspension for at least 30 min at 15,000×g.
9. Load the supernatant onto a column of Ni-NTA resin equilibrated with resuspension buffer. Wash the column with resuspension buffer and 20 mM imidazole until a stable baseline is reached. Elute the fusion protein with a linear gradient over 10 column volumes into elution buffer. The fusion protein usually elutes between 100 and 150 mM imidazole.
10. Mix 3.75 mL of resolving buffer and 3 mL of 40% acrylamide/bis-acrylamide solution (37:1), and add water to a volume of

10 mL in a 50 mL conical flask. Add 100 µL of 10% SDS, 100 µL of ammonium persulfate, and 10 µL of TEMED, and cast gel within a 7.25 × 10 cm × 1 mm gel cassette. Allow space for the stacking gel and gently overlay with ethanol or water.

11. Prepare the stacking gel by mixing 0.63 mL of stacking buffer and 0.5 mL of acrylamide mixture, and adding water to a volume of 5 mL in a 50 mL conical flask. Add 50 µL of 10% SDS, 50 µL of ammonium persulfate, and 5 µL of TEMED. Insert a ten-well gel comb immediately without introducing air bubbles.
12. Add 5 µL SDS loading buffer to a 10 µL sample of each fraction. Load the aliquots on the gel. Electrophorese at 80 V until the samples have entered the gel and then continue at 200 V.
13. Pool fractions of the eluate from the Ni-NTA column containing RNase H and then concentrate and dialyze against the storage buffer (see Note 7).

3.2. RNase H Cleavage

First, choose DNA oligonucleotides which are able to destabilize the tRNA scaffold (see Fig. 1 and Note 2).

1. Mix purified chimeric RNA and the two oligonucleotides (40 mM each, 1:1 ratio with the target RNA), incubate in boiling water (95°C) for 2 min, and then allow to cool down slowly to room temperature (25–30°C). The mix should be prepared in 250 µL maximum volume of water (see Note 8).
2. Add digestion buffer to a final concentration of 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 10 mM $MgCl_2$. Start the cleavage reaction by adding 60 pmol of *E. coli* RNase H

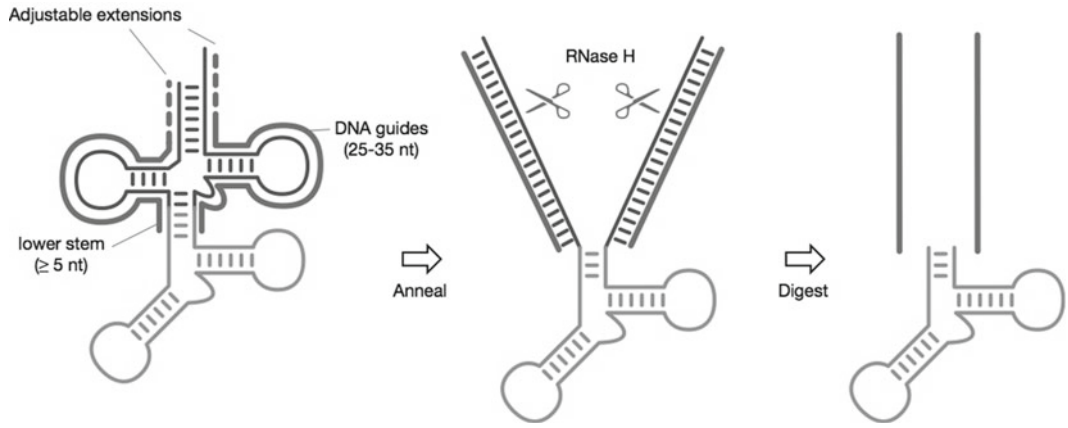

Fig. 1. The different cleavage steps carried out by RNase H. The DNA oligonucleotides are long enough to compete efficiently with the RNA–RNA interactions within the tRNA scaffold and replace them with DNA–RNA pairings after annealing. Typical lengths for these guide DNA oligonucleotides are about 30 nucleotides, but can be varied, as long as they extend at least 5 nucleotides into the lower stem of the tRNA scaffold. After cleavage, the RNA of interest is liberated from the tRNA scaffold.

per nmol of RNA. Incubate for 2 h at 30°C and then stop by addition of an equal volume of 8 M urea (see Note 9).

3. Purify preparative digests by Mono Q ion-exchange chromatography (Roche; see Note 10), under denaturing conditions: 40 mM potassium phosphate, pH 6.5, and 4 M urea (Buffer A). Wash with Buffer A until a stable baseline is reached. Elute the RNA with a 0–0.5 M NaCl gradient in Buffer B (Buffer A + 1 M NaCl) over 10 column volumes and collecting fractions of 5 mL.
4. Prepare the samples for 14% urea–acrylamide gel by mixing 20 μL of each collected fraction with 5 μL of 5× loading buffer.
5. Mix 2 mL of 5 × TBE, 4.25 mL of acrylamide mixture, and 5 g of urea, and add water to a volume of 10 mL in a 50 mL conical flask. Mix to dissolve the urea. Add 100 μL of ammonium persulfate and 10 μL of TEMED, and cast gel within a 7.25 × 10 cm × 1 mm gel cassette. Insert a ten-well gel comb immediately without introducing air bubbles.
6. Assemble the urea–acrylamide gel in the electrophoresis apparatus, fill with 0.5 × TBE running buffer, and load the samples (20 μL each). Electrophorese at 80 V until the samples have entered the gel and then continue at 200 V.
7. Following electrophoresis, pry the gel plates open with the use of a spatula. The gel will remain on one of the glass plates. Gently lift the gel from the glass plate and place it on shrink wrap (see Note 11) and place this on white paper. Reveal the RNA by UV shadowing with the handheld UV lamp placed above the gel.
8. Pool fractions identified as containing the RNA of interest. Dialyze the RNA product successively against 2 L of RNase-free water, then 2 L of 1 M NaCl (see Note 12), and finally with the buffer of your choice.

3.3. Stains-All *Staining*

1. Prepare a polyacrylamide gel for analysis of the RNA product and length marker DNA oligonucleotides, as described in Subheading 3.1, steps 10 and 11.
2. Add 5 μL SDS loading buffer to 10 μL of each DNA oligonucleotide and 10 μL of the RNA product. Load the aliquots on the gel (see Note 13). Electrophorese at 80 V until the samples have entered the gel and then continue at 200 V.
3. After electrophoresis, rinse the gel three times with 25% (v/v) isopropanol followed by washing in 30–50 mL of the same solution on a shaker for 10 min (see Note 14).
4. Replace the isopropanol by 30 mL of *Stains-all* solution. Due to the photosensitivity of *Stains-all*, gels should be incubated in

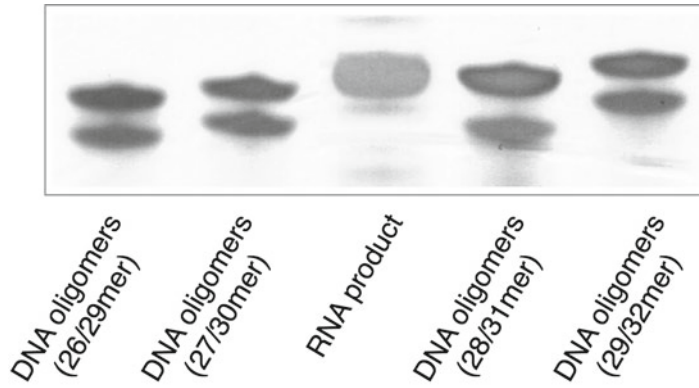

Fig. 2. The length of cleavage product of RNA is analyzed by 12% SDS-PAGE by comparison with DNA oligonucleotide markers. DNA and RNA bands were revealed by *Stains-all* staining (see Note 16). In this example, the estimated length of the RNA product was 31 mer.

a lighttight container on an orbital shaker at room temperature for at least 2 h.

5. Destain the gel with 25% (v/v) isopropanol in a lighttight container on an orbital shaker at room temperature for at least 1 h. Change the destaining solution occasionally (Fig. 2; see Note 15).

Incubation with RNase H will result in specific cleavage of the RNA strand within the DNA:RNA heteroduplexes and hence in complete cleavage of the scaffold. The RNA insert can then be recovered from the mixture and we obtained a single defined product in a quantitative manner.

4. Notes

1. The plasmid can be obtained from the Laboratory of Biological Crystallography and NMR.
2. The DNA oligonucleotides must be long enough to compete efficiently with the RNA–RNA interactions within the tRNA scaffold and replace them with DNA–RNA pairings after annealing. Typical lengths for these guide DNA oligonucleotides are about 30 nucleotides, but can be varied, as long as they extend at least 5 nucleotides into the lower stem of the tRNA scaffold. In practice, we found that they should cover at least the entire TΨC-stem and loop on one side, and the entire D-stem and loop on the other side. Previous reports of specific RNA cleavage using RNase H used modified DNA nucleotides

to improve specificity (7). In the present case, we used standard DNA oligonucleotides.

3. Urea can be dissolved faster provided the water is warmed to about 70–80°C.
4. Use DNA oligonucleotides with length comparable to that of the expected RNA product.
5. Due to the photosensitivity of *Stains-all*, the solution must be shielded from light.
6. Freeze the cell pellet at −80°C until further use (this may be for as long as several months). Perform all of the following procedures at 4°C.
7. Aliquots of RNase H can be stored at −20°C for months (final concentration of 0.25 mM).
8. After this step the RNA is no longer protected; use gloves, RNase-free solutions, and RNase-free materials.
9. Optimization of RNase H reactions: Some optimization must be made each time a new reagent is used (e.g., new plasmid preparation, new RNase H, etc.). In some cases, optimization of the RNase H reaction time is also required to maximize the yield. The critical step is the hybridization of your RNA with the DNA oligonucleotides and it is essential to use DNA oligonucleotides able to destabilize the tRNA scaffold. This step remains, however, very empirical.
10. The preferred ion exchange resin for purifying the cleavage product is MonoQ, as it provides better resolution than Source Q and hence provides better purity.
11. You can use transparent film for wrapping food or any UV transparent support preventing the adsorption of the wet gel on the white paper.
12. The dialysis against 1 M NaCl efficiently removes any remaining urea.
13. Usually denaturing urea polyacrylamide gel electrophoresis employs 8 M urea for DNA or RNA separation in a polyacrylamide gel matrix based on the molecular weight but SDS-PAGE can be used in order to obtain discreet bands.
14. The cycle of rinsing (three times) and washing should be repeated three times. This procedure ensures the removal of all SDS, which, if present, would cause the precipitation of the *Stains-all* dye.
15. Optional: Confirm the estimation of the length of the RNA product by mass spectrometry.
16. *Stains-all* allows differential staining of nucleic acids and proteins. RNA appears in pink and DNA in blue.

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