

Chapter 2

Nanostructured RNAs for RNA Interference

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Abstract

We synthesized three types of nanostructured RNAs that induce RNA interference (RNAi): branched RNAs, dumbbell-shaped RNA, and circular double-stranded RNAs. All three nanostructured RNAs were transformed into double-stranded RNA of approximately 20 base pairs when they were treated with nuclease enzymes such as Dicer. These dsRNA species induced gene silencing when they are were introduced into mammalian cells.

Key words Nanostructured RNA, RNA interference, Small interfering RNA, RNA synthesis

1 Introduction

DNA nanotechnology is a recently emerged technology that can create various self-assembling nanostructures [1–3]. More recently, the marked increase in RNA structural information has led to the development of RNA architectonics, i.e., the scientific study of the principles of RNA architecture with the aim of constructing RNA nanostructures of arbitrary size and shape [4–10]. RNA has more diverse biological functions than does DNA, such as transcription, translation, maturation, and the catalytic activity of ribozymes; therefore, it is very exciting to understand, design, and create new functional RNA nanostructures. To date, various RNA nanostructures have been designed and constructed by assembling small RNA structural motifs [4–10].

In the field of biotechnology related to dsRNA has received much attention after the discovery of RNA interference (RNAi) in 1998 [11]. RNAi is induced by double-stranded RNA of approximately 21 nucleotides (nt), called small interfering (si) RNA and has recently been recognized as a potential therapeutic tool for silencing genes linked to human diseases ([12], *see* Chapters 1, 10, 14, 16). The high sequence specificity and relatively small dose requirement of siRNAs make their use even more attractive for the treatment of various diseases, including cancer [13–15].

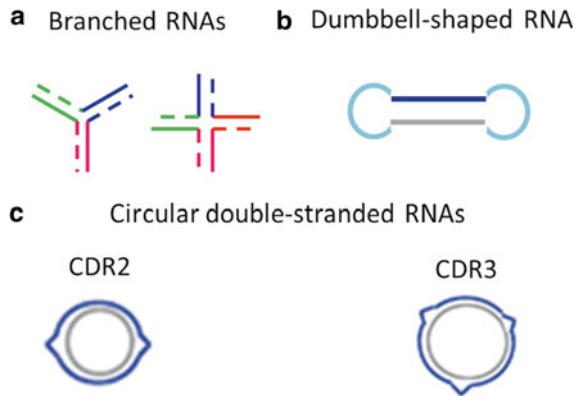


Fig. 1 Design of nanostructured RNAs synthesized in our laboratory. Branched RNAs (a); dumbbell-shaped RNA (b); and circular double-stranded RNAs (c) are shown

However, because natural RNA strands are quickly degraded in biological fluids [16], a method to prolong the RNAi effect is required. If there were a method to enhance the RNAi activity of nontoxic natural RNA strands, it would be very valuable for the therapeutic application of RNAi technology. In addition, formation of effective nanostructure RNAs could enhance their activity to induce RNAi. However, there have been few reports focusing on the relationship between the nanostructure of RNA and its RNAi activity [6, 17].

In this protocol, we described the synthesis of three kinds of nanostructured RNAs that can induce RNAi (Fig. 1). Branched RNAs, containing three- or four-way junctions, were designed by assembling single-stranded RNAs. They comprise three or four siRNA motifs, which were termed trimer RNA or tetramer RNA, respectively [18]. Dumbbell-shaped cyclic RNA, which contains a 23-bp stem (siRNA motif) and two 9-nt loops, are synthesized by an enzymatic method [19, 20]. Circular double-stranded RNA (CDR) comprises two or three siRNA motifs, which were termed CDR2 or CDR3. Each molecule is a catenane, i.e., a molecular architecture consisting of two mechanically interlocked molecules [21].

1.1 Designing Nanostructured RNAs as siRNA Precursors

The nanostructured RNAs contain siRNA motifs in their structures, while branched RNAs contain three or four siRNA motifs as illustrated in Fig. 2a. First, the sequences of canonical siRNA molecules of 21-nt are determined. Second, we elongate their 19 base pair (bp) core sequence with 2-nt on one side by referring to the target mRNA sequence (21-bp blunt dsRNA). We then place the three or four 21-bp dsRNA and connect them at the branch site.

b Dumbbell-shaped RNA

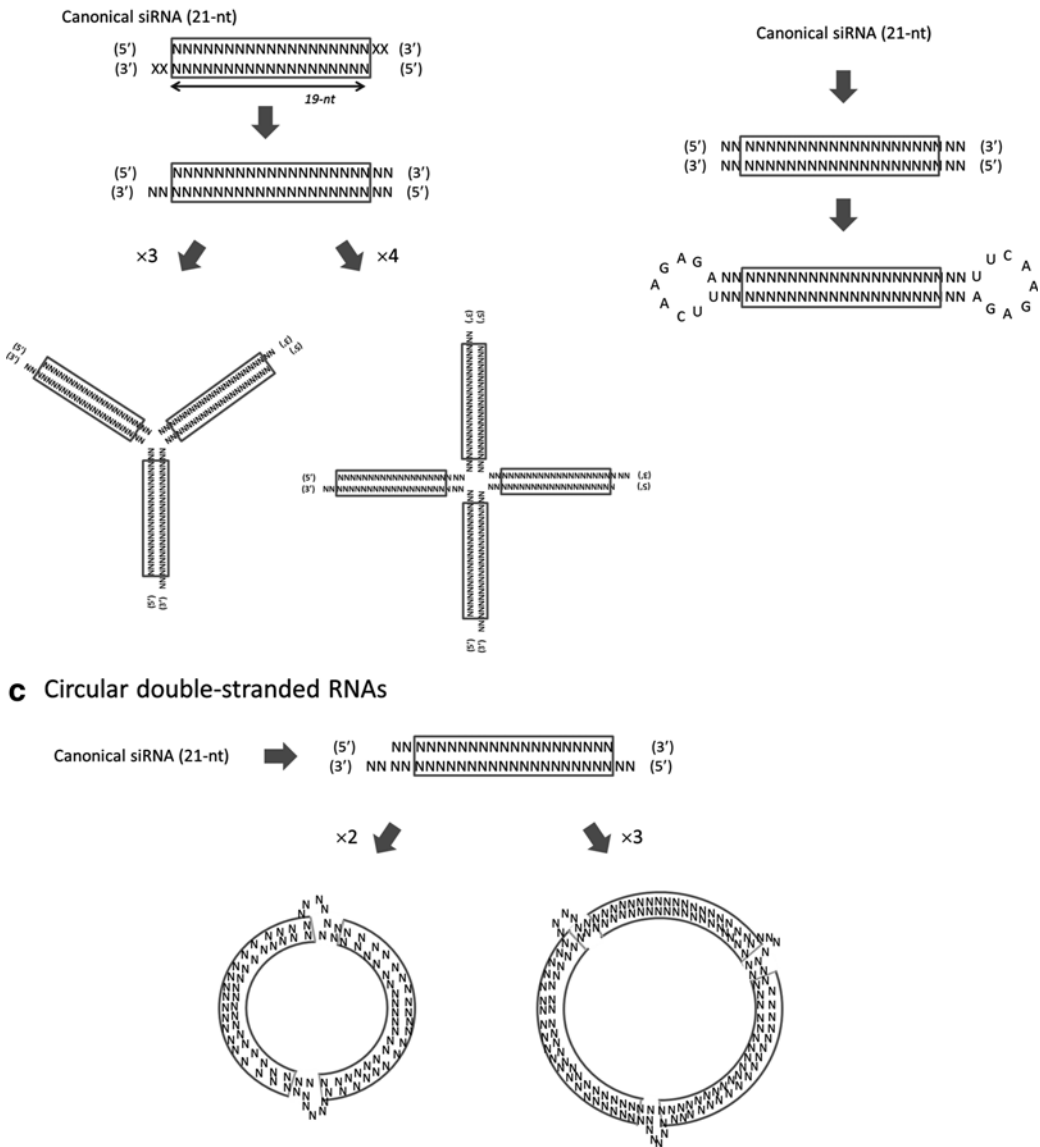


Fig. 2 Schemes for constructing nanostructured RNAs starting from a canonical siRNA molecule. Branched RNAs (**a**); dumbbell-shaped RNA (**b**); and circular double-stranded RNAs (**c**)

The sequence of three or four 48-nt liner RNAs are now determined. We synthesize the 48-nt liner RNAs and anneal them in the buffer (Fig. 3). As for dumbbell-shaped RNAs, they consist of a double-stranded stem region and two signal-stranded loops (Fig. 2b). We optimized the structure in our previous studies [19]. As potent siRNA precursors, RNA dumbbells have a 23-bp stem with two

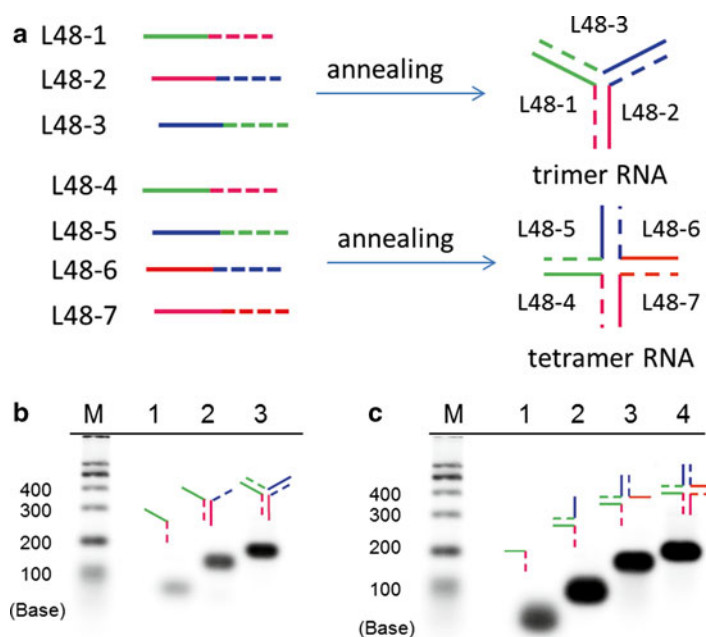


Fig. 3 Formation of branched RNAs. (a) Three or four types of 48-nt RNAs were annealed in aqueous buffer. The samples were analyzed by native agarose gel (3 % agarose in 1× Tris borate buffer) electrophoresis as shown in (b) and (c). (b) Formation of a trimer RNA. Lane 1, L48-1; lane 2, L48-1+L48-2; lane 3, L48-1+L48-2+L48-3 (trimer RNA). (c) Formation of a tetramer RNA. Lane 1, L48-4; lane 2, L48-4+L48-5; lane 3, L48-4+L48-5+L48-6; lane 4, L48-4+L48-5+L48-6+L48-7 (tetramer RNA). The bands were visualized using SYBR Green I

9-nt loops [19, 20]. After the sequence of the siRNA is determined, we elongate the 19-bp core sequence with 2-nt on both sides by referring to the target mRNA sequence (23-bp dsRNA) and connect the two strands by attaching 9-nt loop RNA sequences (5'-UUCAAGAGA-3') on both ends (Fig. 2b). The synthesis of an RNA dumbbell is divided into two components. There are two ways to separate the molecule into two RNA strands. The first choice method comprises separating the RNA dumbbell into two RNA strands by nicking the loops. As an alternative method, two nicks are inserted into the stem region. Each RNA strand is synthesized and purified. Next, these two RNA strands are annealed and ligated using T4 RNA ligase. Finally, denaturing PAGE is used to purify the RNA dumbbell (Fig. 4). As for circular double-stranded RNAs, CDR2 consists of 42-nt circular RNA (sense strand) and 50-nt circular RNA (antisense strand). CDR3 consists of 63-nt circular RNA (sense strand) and 75-nt circular RNA (antisense strand). After determining the sequence of the siRNA, we elongate the 19-bp core sequence with 2-nt on the 5' side of sense and with

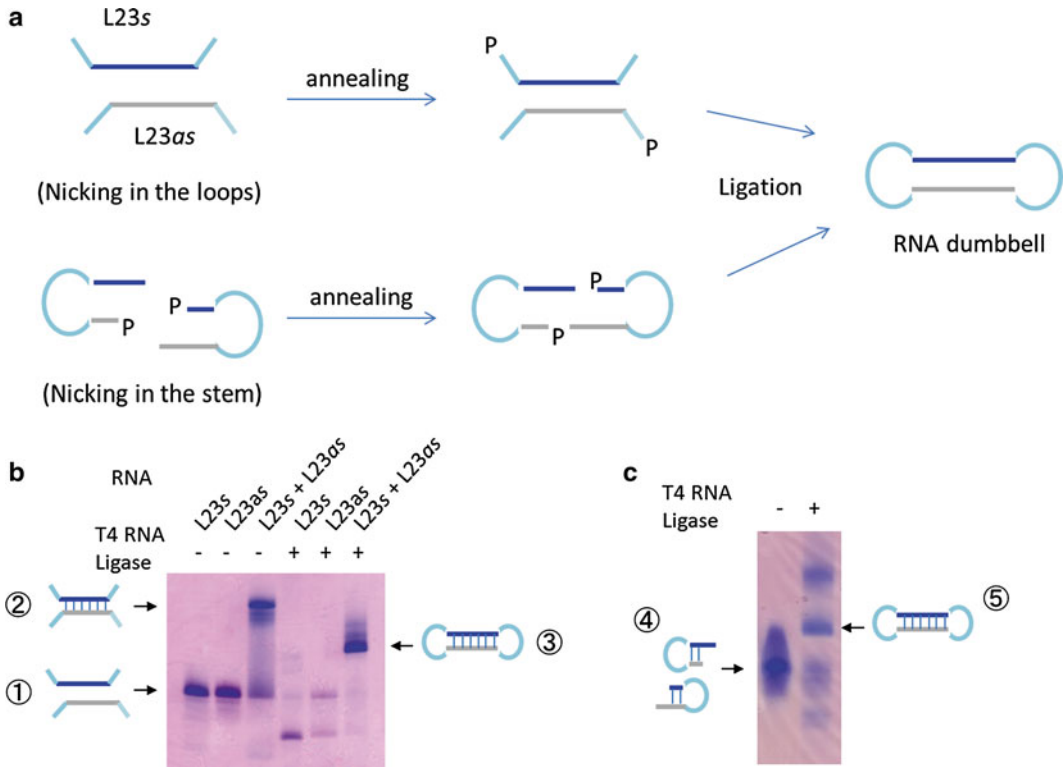


Fig. 4 Synthesis of dumbbell-shaped RNAs. Two RNA strands nicked in their loops (**a, upper**) or in their stems (**a, lower**) were annealed and ligated using T4 RNA ligase. Typical image of synthesized RNA dumbbells by denaturing PAGE analysis (10 % polyacrylamide, 25 % formamide, 7.5 % urea in TBE buffer) (**b, c**). Bands 1–5 are as follows: (1) precursor ssRNAs, denatured; (2) precursor ssRNAs, hybridized; (3) a dumbbell-shaped ligated RNA; (4) precursor hairpin dsRNAs; (5) a dumbbell-shaped ligated RNA

4-nt on the 3' side of antisense, and with 2-nt on the 5' side of antisense by referring to the target mRNA sequence and connect the two (CDR2) or three (CDR3) building units (Fig. 2c).

2 Materials

Prepare all solutions using Milli-Q water (Millipore) and autoclave. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials.

1. Centrifuge tubes (0.6 mL, 1.5 mL, 2.0 mL, 15 mL, and 50 mL).
2. Microcentrifuge tubes with screw caps.
3. Pipette tips (10 μ L, 200 μ L, and 1,000 μ L).
4. Gloves.

5. Glass beakers.
6. 16 °C, 37 °C, and 90 °C heating blocks.
7. Vortex mixer.
8. Freezers and a fridge (−80 °C, −30 °C, and 4 °C).
9. Centrifuge.
10. Incubator.
11. SpeedVac evaporator.

2.1 RNA Synthesis Components

2.1.1 RNA Synthesis Using a DNA Synthesizer

1. 2'-O-Triisopropylsilyloxymethyl (TOM)-Protected RNA Phosphoramidites (A-TOM-CE Phosphoramidite, U-TOM-CE Phosphoramidite, C-TOM-CE Phosphoramidite, G-TOM-CE Phosphoramidite) (Glen Research, USA).
2. RNA controlled-pore glasses (CPGs) (Ac-A-RNA-CPG, U-RNA-CPG, Ac-C-RNA-CPG, Ac-G-RNA-CPG) (Glen Research, USA).
3. Acetonitrile, dehydrated.
4. Deblocking Solution-1 (3 w/v% Trichloroacetic Acid Dichloromethane Solution).
5. Cap A Solution-2 (Tetrahydrofuran–Acetic Anhydride–Pyridine (8:1:1) Solution).
6. Cap B Solution-2 [(10 vol.% 1-Methylimidazole/Tetrahydrofuran Solution)].
7. Oxidizing Solution-1 [0.1 mol/L Iodine, Tetrahydrofuran–Pyridine–Water (7:1:2) Solution].
8. Activator Solution-3 (0.25 mol/L 5-Benzylthio-1H-tetrazole, Acetonitrile Solution).
9. Synthesis column for 0.2 μmol or 1.0 μmol synthesis (Biosearch Technologies Inc.).
10. Frit, Column, 1/8 in × 0.163 (Biosearch Technologies Inc.).
11. Molecular Sieves 3A 1/16.
12. Argon source.
13. DNA synthesizer.

2.1.2 Deprotection of Synthesized RNA

1. Methylamine ammonium solution.
2. Tetrabutylammonium fluoride (TBAF) solution (1 M in THF).
3. 1 M Tris buffer: 1 M Tris–HCl, pH 7.4. Weigh 12.114 g Tris and transfer to the tube (50 mL). Add 90 mL water to the tube. Mix and adjust pH with HCl. Make up to 100 mL with water. Store at 4 °C.
4. Nap-25.
5. Isopropyl alcohol.

6. 3 M sodium acetate (pH 5.2): add 40 mL water to a beaker. Weigh 20.4 g NaOAc + 3H₂O (sodium acetate (NaOAc) + 3H₂O) and transfer the beaker gradually. Mix and adjust pH with glacial acetic acid. Make up to 50 mL with water and autoclave.
7. 80 % (v/v) ethanol.

2.2 Analysis and Purification of RNAs by Polyacrylamide Gel Electrophoresis (PAGE)

1. 0.5 M EDTA (pH 8.0) solution: add 800 mL water to a beaker. Weigh 186.1 g EDTA (ethylenediaminetetraacetic acid + 2Na + 2H₂O) and transfer to the beaker. Add about 5 g of granular NaOH to the beaker to dissolve the EDTA. Mix and adjust pH with 5 N NaOH. Make up to 1 L with water and autoclave.
2. 10× TEB buffer: weigh 108 g Tris and 55 g boric acid and transfer to a 2-L glass beaker. Add to the beaker 40 mL EDTA (0.5 M) and about 800 mL water. Mix using stirrer. Make up to 1 L with water and autoclave.
3. N,N,N',N'-tetramethylethylenediamine (TEMED).
4. Ammonium peroxodisulfate (APS).
5. 20 % denaturing acrylamide gel solution (acrylamide: bisacrylamide = 19:1, 7.5 M urea): weigh 190 g acrylamide, 10 g bisacrylamide, and 450.45 g urea and transfer to a 2-L glass beaker. Add 100 mL of 10× TEB solution and about 500 mL water to the beaker. Mix using stirrer. Make up to 1 L with water. Store in a cool and dark space (*see Note 1*).
6. 2× formamide loading buffer: weigh 100 mg xylene cyanol and 100 mg bromophenol blue and transfer to a 15-mL plastic tube. Add 8 mL formamide and 200 µL of 0.5 M EDTA (pH 8.0). Make up to 10 mL with water. Store at 4 °C (*see Note 2*).
7. 2× formamide loading buffer without dyes: add 8 mL formamide and 200 µL of 0.5 M EDTA (pH 8.0) to a 15-mL plastic tube. Make up to 10 mL with water. Store at 4 °C.
8. Stains-All dye solution: weigh 20 mg Stains-All dye (Sigma-Aldrich) and transfer to a 200 mL of plastic bottle. Add 50 mL of N,N-dimethylformamide (DMF) to dissolve the dye. Then, add 100 mL water to the solution. Store in a cool and dark space.
9. 0.2 M NaCl, 10 mM EDTA (pH 8.0) solution: weigh 2.34 g NaCl and transfer to a beaker. Add 4 mL of 0.5 M EDTA and about 150 mL water to the beaker. Mix using a stirrer. Make up to 200 mL with water. Store at 4 °C.
10. 10 mM EDTA (pH 8.0) solution.
11. Glass plates (20×22 cm).
12. Spacer and comb (1-mm thickness).
13. Large bookbinder clamps.

14. Electrophoresis apparatus.
15. Power supply system.
16. Tray (to stain gels with Stains-All).
17. Plastic container (to stain gels with SYBR Green).
18. Gel photography equipment.
19. Plastic film.
20. Fluorescent thin-layer chromatographic (TLC) plate.
21. Handheld ultraviolet (UV) lamp.
22. Marker pen.
23. Razor blade.
24. Rotating microtube mixer.
25. Filtration device (0.45- μ m pore size).
26. Microcon Ultra YM-3 concentrating and desalting cartridge (Millipore).
27. Sep-Pack desalting cartridge (Waters, UK).
28. UV-Vis spectrometer.

2.3 Synthesis of Branched RNAs, Trimer RNA, and Tetramer RNA

1. RNA oligomers.
2. 5 \times annealing buffer: 100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate.
3. 3 % agarose gel solution: weigh 450 mg Agarose S (Wako pure chemicals) and transfer to a 100-mL beaker. Add TBE buffer to a volume of 15 mL. Mix and dissolve by microwaving.
4. 1 \times TBE buffer.
5. SYBER Green I dye (Cambrex, USA).

2.4 Synthesis of an RNA Dumbbell

1. RNA oligomers.
2. 10 \times T4 RNA Ligation Reaction buffer: 500 mM Tris-HCl (pH 7.5), 100 mM MgCl₂, 1 mM dithiothreitol (DTT), 10 mM ATP.
3. 0.1 % Bovine serum albumin (BSA).
4. 60 % PEG solution: add 600 mg of PEG 6000 and 450 μ L of water to a 2-mL tube. Heat the tube at 90 $^{\circ}$ C to dissolve the PEG 6000.
5. Chloroform.
6. 3 M sodium acetate (pH 5.2).
7. Isopropyl alcohol.
8. 80 % (v/v) ethanol.
9. 7.5 M urea solution (in 1 \times TBE): weigh 450.45 g urea and transfer to a 2-L beaker. Add 100 mL of 10 \times TBE and about

300 mL water to a beaker. Mix using a stirrer. Make up to 1 L with water.

10. 10 % acrylamide gel solution (25 % formamide, 7.5 M urea): add 20 mL of 20 % acrylamide gel solution, 5 mL formamide, and 15 mL 7.5 M urea solution (in 1× TBE) to a beaker. Prepare just before use.

2.5 Synthesis of Circular double- stranded RNAs

1. RNA oligomers.
2. 10× T4 RNA Ligation Reaction buffer: 500 mM Tris-HCl (pH 7.5), 100 mM MgCl₂, 1 mM dithiothreitol (DTT), 10 mM ATP.
3. 0.1 % BSA.
4. 60 % PEG solution.
5. Chloroform.
6. 3 M sodium acetate (pH 5.2).
7. Isopropyl alcohol.
8. 80 % (v/v) ethanol.
9. 7.5 M urea solution (in 1× TBE).
10. 10 % acrylamide gel solution (25 % formamide, 7.5 M urea).
11. 40 % non-denature acrylamide gel solution: weigh 190 g acrylamide and 10 g bisacrylamide and transfer to a 1-L glass beaker. Add 50 mL of 10× TEB and approximately 250 mL water to the beaker. Mix using a stirrer. Make up to 500 mL with water. Store it in a cool and dark space.

3 Methods

All procedures are carried out at room temperature unless otherwise specified.

3.1 RNA Synthesis Using a DNA/RNA Synthesizer

1. Calculate the amount of phosphoramidites and activator required to assemble the desired sequence by multiplying the following by the number of nucleotides in the sequence, and adding an extra milligram of phosphoramidites to give enough material to purge the lines of the synthesizer (*see Note 3*).
2. Use a syringe to add the amount of dry acetonitrile required to obtain 0.05 M phosphoramidite. Allow the solids to dissolve. Add beads of 3A molecular sieves to the phosphoramidite solution.
3. Calculate the required amount of CPG support according to its individual loading and the synthesis scale. Load an empty synthesis column with the RNA CPG. (0.2 μmol scale; A CPG 8.7 mg, U CPG 6.9 mg, C CPG 5.7 mg, G CPG 7.7 mg. 1.0 μmol scale; fivefold amount of 0.2 μmol).

4. Connect the column, reagents, amidites, and argon to the synthesizer.
5. Create methods for the assembly of the desired sequence. Use a 3-min coupling time for RNA monomers when using 5-Benzylthio-1H-tetrazole as the activator.
6. Choose the option “DMTr off” in the setup menu.
7. Purge the lines of the synthesizer with all solutions and solvents three times.
8. Click the start button. Do not leave the DNA synthesizer until the second cycle is finished and check the color (changes to orange) in the detritylation step.
9. Check the synthesis yield on the monitor.
10. Remove the synthesis column from the synthesizer and thoroughly air-dry the support in the column.

3.2 Deprotection of Synthesized RNAs

1. Open the synthesis column and pour the support into a 2-mL centrifuge tube with a screw cap.
2. Add AMA solution (mix equal volumes of 28 % ammonium hydroxide and 40 % aqueous methylamine) to the tube (1 mL for a 0.2 μ mol, 1.5 mL for a 1 μ mol synthesis), seal the tube, and incubate it for 6 h at 35 °C, or at room temperature overnight.
3. Cool the tube on ice then open it cautiously. The use of sterile conditions from this point forward is essential. Filter the supernatant solution from the support using a Pasteur pipette filled with a little cotton. Rinse the support with 3 times 1 mL of sterile water.
4. Evaporate the combined solutions to dryness in a vacuum concentrator.
5. Add 1 mL TBAF solution to the residue and warm the tube at 50 °C with shaking for 10 min to dissolve the residue. Allow it to cool to 35 °C and incubate it for at least 6 h or overnight.
6. Add 1 mL of 1 M Tris-HCl buffer (pH 7.4) solution to the tube and shake well. This step also removes the 2'-hemiacetals.
7. Remove the THF in a vacuum concentrator for 30 min. There is no need to evaporate the solution to dryness, although this is not harmful.
8. Add 1 mL of sterile water to the solution to give a final volume of approximately 2 mL.
9. Having equilibrated a NAP-25 column with 25 mL of water, add the oligonucleotide solution to the column.
10. Allow the sample solution to enter the gel completely and add a further 0.5 mL of water.

11. Elute the desalted oligo with 3 mL of water. Eluting with a smaller volume is preferred because a larger volume contaminates the oligonucleotides with salts.
12. Concentrate the oligonucleotide solution from 3 mL to 200 μ L. Transfer the concentrated oligonucleotide solution to a 1.5-mL tube.
13. Add 400 μ L of isopropyl alcohol and 20 μ L of 3 M sodium acetate solution to the tube. Cool the tube at -20°C for 20 min.
14. Centrifuge the tube for 30 min at $15,000\times g$, 4°C . Remove the supernatant and add 300 μ L of 80 % ethanol to rinse the pellet. Centrifuge the tube for 5 min at $15,000\times g$, 4°C .
15. Discard the supernatant. Dry the pellet by leaving the lid of the tube open for 15 min at room temperature.
16. Dissolve the pellet in 100 μ L of water.
17. Measure the absorbance at 260 nm and prepare a 50 μ M oligo solution.

3.3 PAGE Analysis (10 % Polyacrylamide)

1. Clean the glass gel plates with water and 70 % ethanol. Set the spacer in the plates and clip the plates together.
2. Mix 20 mL of 20 % acrylamide gel solution and 20 mL of urea solution (in $1\times$ TBE buffer) in a 100-mL conical flask. Add 400 μ L of 10 % (w/v) APS and 40 μ L of TEMED.
3. Pour it quickly into the mold of plates. Insert a gel comb immediately without introducing air bubbles. Place the plates on the bench until the gel polymerizes (about 1 h).
4. Set up the gel using TBE as the running buffer. Wash the wells using running buffer. Prerun the gel at 15 W (constant wattage) for 15 min.
5. Add 4 μ L of $2\times$ formamide loading buffer to the 4 μ L of the solution prepared Subheading 3.2, step 17.
6. Wash the well again and load the sample to the gel. Electrophorese at 20 W until the dye front (from the BPB dye in the samples) has reached the bottom of the gel.
7. Following electrophoresis, pry the gel plates open using a spatula. The gel remains on one of the glass plates.
8. Cut excess gel using a spatula.
9. Transfer carefully to a container with stains-all solution using a plastic sheet.
10. Incubate at room temperature for 30 min.
11. Wash the gel $2\times$ with water.
12. Wrap the gel with plastic film. Photograph the gel.

3.4 PAGE Purification

1. Add an equal volume of 2× formamide loading buffer without dye to the RNA precipitated in Subheading 3.2, step 17. Repeat Subheading 3.3, steps 1–7 of denaturing PAGE.
2. Wrap the gel in plastic film. Place the gel on the fluorescent TLC plate. Visualize the RNA in the gel using a handheld UV lamp. Mark the bands with a marker pen.
3. Cut the bands out with a razor blade. Transfer the separated the gel into a 15-mL tube.
4. Add 2 mL of 0.2 M NaCl containing 10 mM EDTA to the 15-mL tube.
5. Crush the gel with a 10-mL disposable plastic pipette.
6. Add a further 5 mL of 0.2 M NaCl containing 10 mM EDTA to the tube.
7. Rotate the tube in a rotating tube mixer at room temperature overnight to extract the RNA from the gel.
8. Transfer the supernatant to a new tube. Add 5 mL of 0.2 M NaCl containing 10 mM EDTA to the tube containing the gel and rotate in a rotating tube mixer at room temperature for 3 h. Transfer the supernatant to the tube containing the 1st supernatant (termed the extract).
9. Desalt the extract using a Sep-Pak cartridge as follows:
 - (a) Mix 12 mL of the gel extract and 0.5 mL of 2 M TEAA buffer.
 - (b) Set a Sep-Pak cartridge into a 10-mL syringe.
 - (c) Wash the Sep-Pak cartridge with 2× 10 mL of acetonitrile and 2× 5 mL of water.
 - (d) Equilibrate a Sep-Pak cartridge with 2 mL of TEAA buffer. TEAA buffer should leave a 2-mm gap at the top of the cartridge.
 - (e) Load the buffered gel extract to the equilibrated cartridge. Repeat this step four to five times.
 - (f) Wash the cartridge with 3 times with 10 mL of water.
 - (g) Elute with 6 mL of 50 % acetonitrile and 3 mL of 100 % acetonitrile.
10. Concentrate the solution to 200 μ L. Transfer the concentrated solution to a 1.5-mL tube.
11. Add 400 μ L of isopropyl alcohol and 20 μ L of 3 M sodium acetate solution to the tube. Cool the tube at -20°C for 20 min.
12. Centrifuge the tube for 30 min at $15,000\times g$, 4°C . Remove the supernatant and add 300 μ L of 80 % ethanol to rinse the pellet. Centrifuge the tube for 5 min at $15,000\times g$, 4°C .
13. Discard the supernatant. Dry the pellet by leaving the lid of the tube open for 15 min at room temperature.

14. Dissolve the pellet in 10 μL of water.
15. Measure the absorbance at 260 nm using a UV-Vis spectrometer to determine the concentration of RNA and prepare a 50 μM oligo solution. Store at $-30\text{ }^{\circ}\text{C}$.

3.5 Construction Branched RNAs (Trimer RNA, Tetramer RNA)

1. Synthesize the RNAs (L48-1, 2, 3, 4, 5) using a DNA synthesizer and perform PAGE purification as in steps in Subheadings 3.1–3.4.
2. Mix 2 μL of 50 μM three (L48-1, 2, 3) or four (L48-2, 3, 4, 5) RNAs, 4 μL of 5 \times annealing buffer and 10 or 8 μL of water in a 1.5 mL tube (total volume 20 μL). Heat the tube at $90\text{ }^{\circ}\text{C}$ for 3 min. Cool the tube in a heating block to room temperature. Analyze the mixture by 3 % agarose gel electrophoresis using TBE buffer. Electrophorese at 100 V until the dye front (from the BPB dye in the samples) has reached the bottom of the gel. Subsequently, transfer the gel to a container with 2 μL of SYBR Green II in 20 mL TBE buffer and incubate at room temperature for 30 min. Scan the gel to visualize the bands on a Bio-Rad Molecular Imager FX (Bio-Rad, CA). Wash the stage of the Imager with water and methanol before and after use.

3.6 Synthesis of Dumbbell-Shaped Cyclic RNAs

1. Synthesize the RNAs (L32s, L32as) using a DNA synthesizer and purify by PAGE as in Subheadings 3.1–3.4. Perform 5'-phosphorylation during synthesis using a chemical phosphorylation reagent.
2. Add 40 μL of 50 μM L32s, 40 μL of 50 μM L32as, and 100 μL of 10 \times buffer (500 mM Tris-HCl (pH 7.5), 100 mM MgCl_2 , 10 mM DTT, and 10 mM ATP) and 333.3 μL of water to a tube. Heat the tube at $90\text{ }^{\circ}\text{C}$ for 3 min. Cool the tube in a heating block to room temperature.
3. Add 10 μL of 40 units/ μL T4 RNA ligase, 416.7 μL of 60 % PEG 6000, 60 μL of 0.1 % BSA to the tube.
4. Incubate the tube at $16\text{ }^{\circ}\text{C}$ overnight.
5. Add 1 mL of chloroform.
6. Mix the tube well.
7. Centrifuge the tube for 5 min at $15,000\times g$, room temperature.
8. Transfer the aqueous layer to a new tube.
9. Repeat steps 5–8 twice.
10. Add 1 mL of isopropyl alcohol and 100 μL of 3 M sodium acetate solution to the tube. Cool the tube at $-30\text{ }^{\circ}\text{C}$ for at least 3 h.
11. Centrifuge the tube for 30 min at $15,000\times g$, $4\text{ }^{\circ}\text{C}$. Remove the supernatant and add 300 μL of 80 % ethanol to rinse the pellet. Centrifuge the tube for 5 min at $15,000\times g$ at $4\text{ }^{\circ}\text{C}$.

12. Discard the supernatant. Dry the pellet by leaving the lid of the tube open for 15 min at room temperature.
13. Dissolve the pellet in 30 μ L of water.
14. Perform 10 % PAGE analysis (*see* Subheading 3.3).
15. Perform 10 % PAGE purification as follows:
 - (a) *See* Subheading 3.4, steps 1 and 2
 - (b) Excise the bands with a razor blade. Transfer into a 2-mL tube.
 - (c) Crush the gel with a disposable 200- μ L pipette tip. Add 1 mL of 10 mM EDTA (pH 8.0) to the tube.
 - (d) Rotate the tube in a rotating tube mixer at room temperature for about 6 h to extract the RNA from the gel.
 - (e) Centrifuge the tube for 5 min at 15,000 $\times g$, room temperature. Transfer the supernatant to a new tube and store at -30°C . Add 0.5 mL of EDTA to the tube containing the gel and rotate in a rotating tube mixer at room temperature for about 6 h. Repeat this step once more.
16. Filter the supernatant with a Millex filter.
17. Concentrate and desalt the extract using a Microcon Ultracel YM-3 cartridge (500 μ L of the solution is reduced to ~ 100 μ L). Add 400 μ L of water and filter the solution again to approximately 50 μ L. Recover the sample in a tube supplied by the manufacturer, and then transfer the sample into a 1.5-mL tube. Add 100 μ L of isopropyl alcohol and 5 μ L of 3 M sodium acetate solution to the tube. Cool the tube at -30°C for at least 3 h and then centrifuge the tube for 20 min at 15,000 $\times g$, 4°C . Remove the supernatant and add 100 μ L of 80 % ethanol to rinse the pellet. Centrifuge the tube for 5 min at 15,000 $\times g$ at 4°C .
18. Discard the supernatant. Dry the pellet by leaving the lid of the tube open for 15 min at room temperature. Dissolve the pellet in a small volume of water (typically 100 μ L).
19. Measure the absorbance at 260 nm using a UV-Vis spectrometer to determine the concentration of the RNA.

3.7 Synthesis of Circular Double- Stranded RNAs (CDR2)

1. Synthesize the RNAs (L21s, L25as) using a DNA synthesizer and perform PAGE purification as in Subheadings 3.1–3.4. In this case, 5'-phosphorylation was performed on the synthesizer using a chemical phosphorylation reagent.
2. Add 500 μ L of 10 μ M L21s, 6.25 μ L of 40 units/ μ L T4 RNA ligase, 2,100 μ L of 60 % PEG 6000, 300 μ L of 0.1 % BSA, 500 μ L of 10 \times buffer (500 mM Tris-HCl (pH 7.5), 100 mM MgCl_2 , 10 mM DTT, and 10 mM ATP) to a tube. Make up to 5.0 mL with water.
3. Incubate the tube at room temperature overnight.

4. Add 5 mL of chloroform.
5. Mix the tube well.
6. Centrifuge the tube for 5 min at $7,000\times g$.
7. Transfer the aqueous layer to a new tube.
8. Repeat **steps 4–7**.
9. Transfer $\sim 900\ \mu\text{L}$ of the aqueous layer to a new tube.
10. Add 1 mL of chloroform.
11. Repeat **steps 5–7**.
12. Add 1 mL of isopropyl alcohol and $100\ \mu\text{L}$ of 3 M sodium acetate solution to the tube. Cool the tube at $-30\ ^\circ\text{C}$ for at least 3 h.
13. Centrifuge the tube for 20 min at $15,000\times g$, $4\ ^\circ\text{C}$. Remove the supernatant and add 1 mL of 80 % ethanol to rinse the pellet. Centrifuge the tube for 5 min at $15,000\times g$, $4\ ^\circ\text{C}$.
14. Discard the supernatant. Dry the pellet by leaving the lid of the tube open for 15 min at room temperature.
15. Dissolve the pellet in $20\ \mu\text{L}$ of water and gather divided RNAs together one tube.
16. Perform 10 % PAGE analysis (*see* Subheading 3.3)
17. Perform 10 % PAGE purification (*see* Subheading 3.6, **step 14**). And then concentrate and desalt the extract using a Microcon Ultracel YM-3 cartridge ($500\ \mu\text{L}$ of the solution is reduced to $\sim 100\ \mu\text{L}$). Add $400\ \mu\text{L}$ of water and filter the solution again to about $50\ \mu\text{L}$. Recover the sample in a tube supplied by the manufacturer, and then transfer the sample into a 1.5-mL tube. Add $100\ \mu\text{L}$ of isopropyl alcohol and $5\ \mu\text{L}$ of 3 M sodium acetate solution to the tube. Cool the tube at $-30\ ^\circ\text{C}$ for at least 3 h. Centrifuge the tube for 20 min at $15,000\times g$, $4\ ^\circ\text{C}$. Remove the supernatant and add $100\ \mu\text{L}$ of 80 % ethanol to rinse the pellet. Centrifuge the tube for 5 min at $15,000\times g$, $4\ ^\circ\text{C}$. Discard the supernatant. Dry the pellet by leaving the lid of the tube open for 15 min at room temperature. And finally, dissolve the pellet in a small volume of water (typically $100\ \mu\text{L}$).
18. Measure the absorbance at 260 nm using a UV-Vis spectrometer to determine the concentration of the RNA.
19. Add $20\ \mu\text{L}$ of $50\ \mu\text{M}$ C42s, $80\ \mu\text{L}$ of $50\ \mu\text{M}$ L25as, $100\ \mu\text{L}$ of $10\times$ buffer ($500\ \text{mM}$ Tris-HCl (pH 7.5), $100\ \text{mM}$ MgCl_2 , $10\ \text{mM}$ DTT, and $10\ \text{mM}$ ATP), and $317.5\ \mu\text{L}$ of water to a tube. Heat the tube at $90\ ^\circ\text{C}$ for 3 min. Cool the tube in a heating block to room temperature.
20. Add $2.5\ \mu\text{L}$ of 40 units/ μL T4 RNA ligase, $420\ \mu\text{L}$ of 60 % PEG 6000, $60\ \mu\text{L}$ of 0.1 % BSA to the tube.
21. Incubate the tube at room temperature overnight.

22. Add 800 μL of chloroform.
23. Mix the tube well.
24. Centrifuge the tube for 5 min at $7,000\times g$, room temperature.
25. Transfer the aqueous layer to a new tube.
26. Add 1 mL of chloroform.
27. Mix the tube well.
28. Centrifuge the tube for 5 min at $7,000\times g$, room temperature.
29. Transfer the aqueous layer to a new tube.
30. Add 1 mL of isopropyl alcohol and 90 μL of 3 M sodium acetate solution to the tube. Cool the tube at $-30\text{ }^{\circ}\text{C}$ for at least 3 h.
31. Centrifuge the tube for 30 min at $15,000\times g$, $4\text{ }^{\circ}\text{C}$. Remove the supernatant and add 300 μL of 80 % ethanol to rinse the pellet. Centrifuge the tube for 5 min at $15,000\times g$, $4\text{ }^{\circ}\text{C}$.
32. Discard the supernatant. Dry the pellet by leaving the lid of the tube open for 15 min at room temperature.
33. Dissolve the pellet in 30 μL of water.
34. Perform 10 % denaturing PAGE analysis (*see* Subheading 3.3).
35. Perform 10 % denaturing PAGE purification (*see* Subheading 3.6, step 14).
36. Repeat **steps 18 and 19**.

3.8 Synthesis of Circular Double- Stranded RNAs (CDR3)

1. Synthesize the RNAs (L63s, L25as) using a DNA synthesizer and perform PAGE purification as in **steps 1–19**. In this case, 5'-phosphorylation was performed on the synthesizer using a chemical phosphorylation reagent.
2. Add 5 μL of 50 μM L63s, 0.667 μL of 40 units/ μL T4 RNA ligase, 420 μL of 60 % PEG 6000, 60 μL of 0.1 % BSA, 100 μL of 10 \times buffer (500 mM Tris-HCl (pH 7.5), 100 mM MgCl_2 , 10 mM DTT, and 10 mM ATP) to a tube. Make up to 1 mL with water.
3. Incubate the tube at room temperature overnight.
4. Add 800 μL of chloroform.
5. Mix the tube well.
6. Centrifuge the tube for 5 min at $7,000\times g$, room temperature.
7. Transfer the aqueous layer to a new tube.
8. Add 1 mL of chloroform.
9. Mix the tube well.
10. Centrifuge the tube for 5 min at $7,000\times g$, room temperature.
11. Transfer the aqueous layer to a new tube.
12. Repeat **steps 8–11**.

13. Add 1 mL of isopropyl alcohol and 100 μ L of 3 M sodium acetate solution to the tube. Cool the tube at -30°C for at least 3 h.
14. Centrifuge the tube for 20 min at $15,000\times g$, 4°C . Remove the supernatant and add 1 mL of 80 % ethanol to rinse the pellet. Centrifuge the tube for 5 min at $15,000\times g$, 4°C .
15. Discard the supernatant. Dry the pellet by leaving the lid of the tube open for 15 min at room temperature.
16. Dissolve the pellet in 20 μ L of water.
17. Measure the absorbance at 260 nm using a UV-Vis spectrometer to determine the concentration of the RNA.
18. Perform 10 % native PAGE analysis (*see* Subheading 3.3).
19. Add 70 μ L of 10 μM C63s, 10 μ L of 300 μM L25as and 75 μ L of 10 \times buffer (500 mM Tris-HCl (pH 7.5), 100 mM MgCl_2 , 10 mM DTT, and 10 mM ATP) and 225 μ L of water to a tube. Heat the tube at 90°C for 3 min. Cool the tube in a heating block to room temperature.
20. Add 10 μ L of 30 units/ μ L T4 RNA ligase, 315 μ L of 60 % PEG 6000, 45 μ L of 0.1 % BSA to the tube.
21. Incubate the tube at room temperature overnight.
22. Add 1 mL of chloroform.
23. Mix the tube well.
24. Centrifuge the tube for 5 min at $7,000\times g$, room temperature.
25. Transfer the aqueous layer to a new tube.
26. Repeat **steps 22–25** twice.
27. Add 750 μ L of isopropyl alcohol and 75 μ L of 3 M sodium acetate solution to the tube. Cool the tube at -30°C for at least 3 h.
28. Centrifuge the tube for 30 min at $15,000\times g$, 4°C . Remove the supernatant and add 300 μ L of 80 % ethanol to rinse the pellet. Centrifuge the tube for 5 min at $15,000\times g$, 4°C .
29. Discard the supernatant. Dry the pellet by leaving the lid of the tube open for 15 min at room temperature.
30. Dissolve the pellet in 30 μ L of water.
31. Perform 10 % denaturing PAGE analysis (*see* Subheading 3.3)
32. Perform 10 % denaturing PAGE purification (*see* Subheading 3.6, **step 14**)
33. Concentrate and desalt the extract using a Microcon Ultracel YM-3 cartridge (500 μ L of the solution is reduced to ~ 100 μ L). Add 400 μ L of water and filter the solution again to about 50 μ L. Recover the sample in a tube supplied by the manufacture, and then transfer the sample into a 1.5-mL tube.

Add 100 μL of isopropyl alcohol and 5 μL of 3 M sodium acetate solution to the tube. Cool the tube at $-30\text{ }^{\circ}\text{C}$ for at least 3 h. Centrifuge the tube for 20 min at $15,000\times g$, $4\text{ }^{\circ}\text{C}$. Remove the supernatant and add 100 μL of 80 % ethanol to rinse the pellet. Centrifuge the tube for 5 min at $15,000\times g$, $4\text{ }^{\circ}\text{C}$. Discard the supernatant. Dry the pellet by leaving the lid of the tube open for 15 min at room temperature and then dissolve the pellet with a small volume of water (typically 100 μL). Measure the absorbance at 260 nm using a UV-Vis spectrometer to determine the concentration of the RNA.

Figure 5 illustrates the assembly of structured double-stranded RNAs.

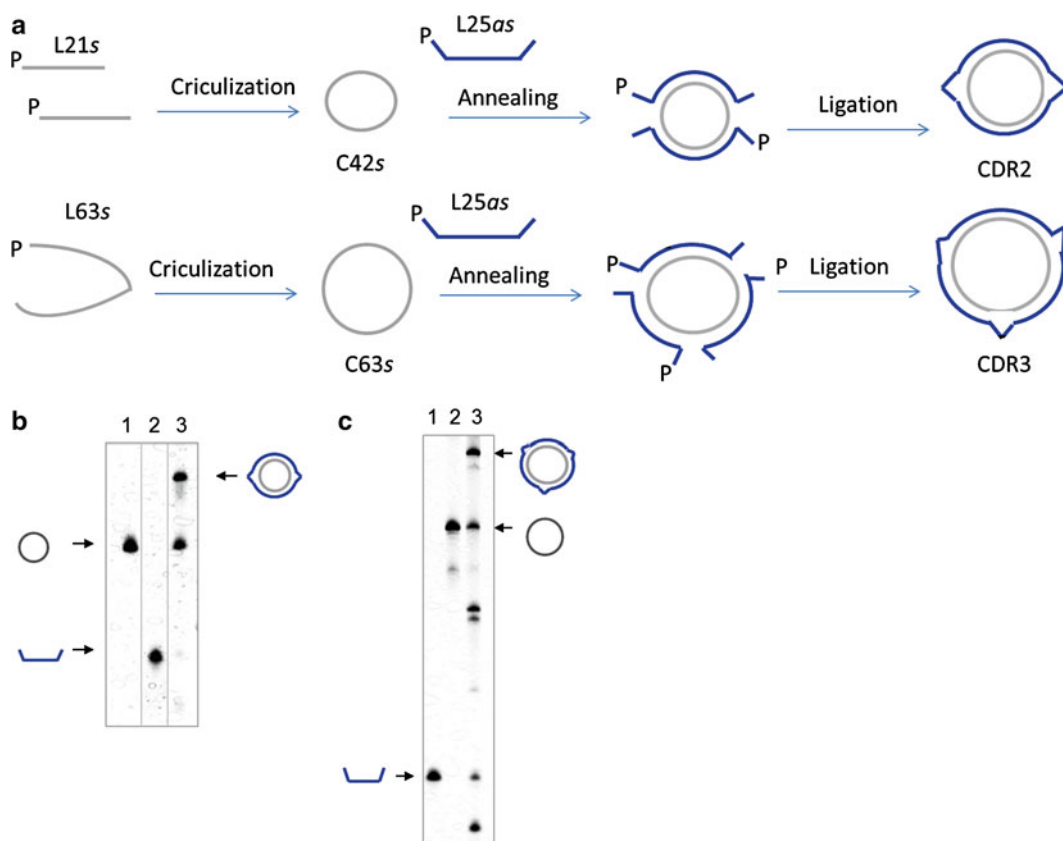


Fig. 5 Stepwise assembly of structured circular double-stranded RNAs. CDR2 (**a**, upper), CDR3 (**a**, lower) were constructed as shown. All RNA oligos to be ligated were 5'-phosphorylated. T4 RNA ligase was used in all reactions. Synthesis of double-stranded circular RNAs by template-mediated ligation. Ligation reactions were analyzed by 10 % denaturing PAGE (7 M urea, 25 % formamide in TBE) and visualized by SYBR Green II. (**b**) Synthesis of CDR2. Lane 1, C42s; lane 2, L25as; lane 3, C42s + L25as + ligase. (**c**) Synthesis of CDR3. Lane 1, L25as; lane 2, C63s; lane 3, L25as + C63s + ligase

4 Notes

1. Wear a mask when weighing acrylamide. Transfer the weighed acrylamide to the cylinder inside the fume hood and mix on a stirrer placed inside the hood. Unpolymerized acrylamide is a neurotoxin and care should be exercised to avoid skin contact.
2. Formamide is teratogenic. Wear appropriate gloves and safety glasses.
3. Store RNA amidites at -30°C before dissolving them in acetonitrile. After dissolving in acetonitrile, store at -80°C .

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