# Supporting Information

## Synthesis and Characterization of Small Circular Double-Stranded RNAs

Naoko Abe,[1] Hiroshi Abe,*[1] Takahito Ohshiro,[2] Yuko Nakashima,[1] Mizuo Maeda,[2] and Yoshihiro Ito*[1]

[1] Nano Medical Engineering Laboratory, RIKEN Advanced Science Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198 (Japan)
FAX: +81(0)48-467-9300
E-mail: h-abe@riken.jp; y-ito@riken.jp
[2] Bioengineering Laboratory, RIKEN Advanced Science Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198 (Japan)

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***Table S1.*** Sequences of single-stranded RNAs used in this study.

| Name* | Sequence (5′ to 3′)** |
|---|---|
| L21*s* | pUUCUUACGCUGAGUACUUCGA |
| L42*s* | pUUCUUACGCUGAGUACUUCGA-UUCUUACGCUGAGUACUUCGA |
| C42*s* | Circular form of L42*s* |
| L63*s* | pUUCUUACGCUGAGUACUUCGA-UUCUUACGCUGAGUACUUCGA-UUCUUACGCUGAGUACUUCGA |
| C63*s* | Circular form of L63*s* |
| L25*as* | pUCUCGAAGUACUCAGCGUAAGUGAA |
| C50*as* | -UCUCGAAGUACUCAGCGUAAGUGAA-UCUCGAAGUACUCAGCGUAAGUGAA-,Circularized dimeric form of L25*as* |

*The first letter in the name, L = liner; C = circular. Two-digit number signifies its nucleotide length. The last letter(s), *s* = sense; *as*= antisense.

** Letter p on 5′ -end means it is phosphorylated. Underlined bases are expected to build bulged regions in its resulting double-stranded circles because they do not have a base-paring partner in the complementary sequences.

***Table S2.*** MALDI-TOF MS analysis of single-stranded RNAs. Data were obtained by microflex MALDI-TOF mass spectrometer (Bruker Daltonics) at RIKEN BSI RRC, by positive mode using 3-hydroxypicolinic acid (HPA) as a matrix.

| Name of RNA | $MH^+$ | | |
|---|---|---|---|
| | calcd | found | difference |
| L21*s* | 6,691.9 | 6,690.7 | -1.2 |
| L42*s* | 13,364.9 | 13,365.0 | +0.1 |
| C42*s* | 13,346.8 | 13,337.7 | -9.1 |
| L25*as* | 8,086.8 | 8,084.8 | -2.0 |
| C50*as* | 16,136.7 | 16,133.2 | -3.5 |

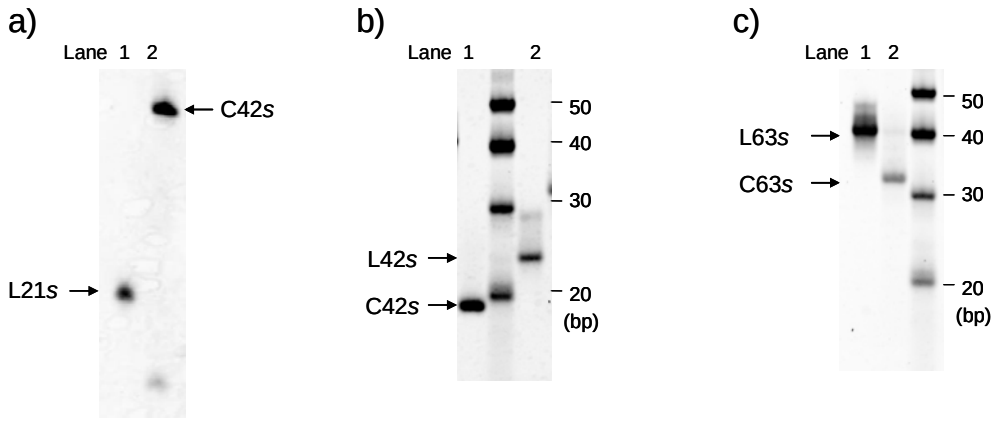


***Figure S1.*** PAGE analysis of the ligation reaction to synthesize circular ssRNAs. SYBR Green I was used to visualize non-denaturing PAGE [10% PAGE in 89 mM Tris-borate, 2 mM EDTA, pH 8.3 (TBE)]. SYBR Green II for denaturing PAGE (10% PAGE, 7 M urea, 25% formamide in TBE). siRNA Ladder Marker (Takara bio, Japan) was used as a dsRNA size marker.

a) Denaturing PAGE analysis. lane1, L21*s*; lane 2, L21*s* + T4 RNA ligase. b) Non-denaturing analysis of C42*s* (lane 1, after being isolated) and L42*s* (lane 2). Difference in migration can be observed. c) Non-denaturing analysis of the ligation reaction. Lane1, L63*s*; lane 2, L63*s* + T4 RNA ligase. Difference in migration after being circularized can be observed.

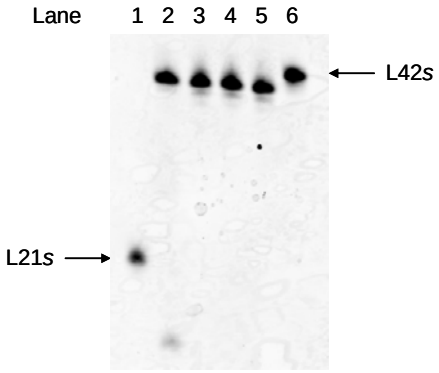


***Figure S2.*** Denaturing PAGE (10% PAGE, 7 M urea, 25% formamide in TBE) analysis of ligation reactions to obtain C42s from L21s or from L42s. Gel was visualized by SYBR Green II staining. Lane 1, L21*s*. Lane 2, L21*s* + T4 RNA ligase. Lanes 3-5, L42*s* + T4 RNA ligase. Lane 6, L42*s*.

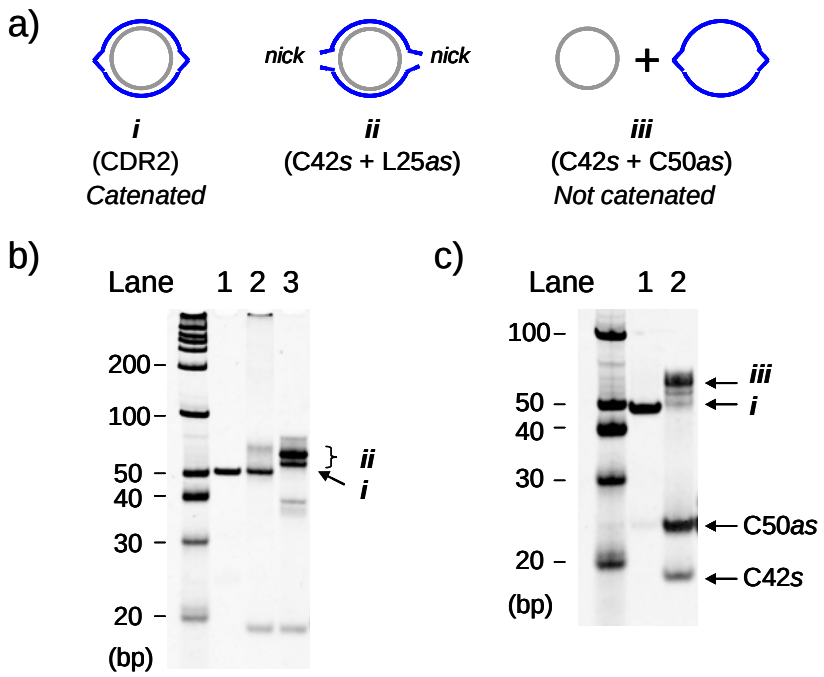


***Figure S3.*** Non-denaturing PAGE analysis of CDR2 and its related structures. Analyzed by non-denaturing PAGE and compared their difference in migration. PAGE concentration; 15% in (b), 10% in (c). Gels were stained with SYBR Green I. siRNA Ladder Marker (Takara bio, Japan) was used as a dsRNA size marker. a) related structures of RNAs. b) Ligation reaction to synthesize CDR2. Lane 1, CDR2 after being purified by denaturing PAGE. Lane 2, reaction mixture to synthesize CDR2; C42*s* + L25*as* + T4 RNA ligase, that correspond to lane 6 in Figure 3 **a**. Lane 3, reaction mixture to synthesize CDR2 without ligase; C42*s* +L25*as*, that correspond to lane 5 in Figure 3a. c) Effect of catenane formation in migration. Lane 1, CDR2. Lane 2, C42*s* + C50*as*.

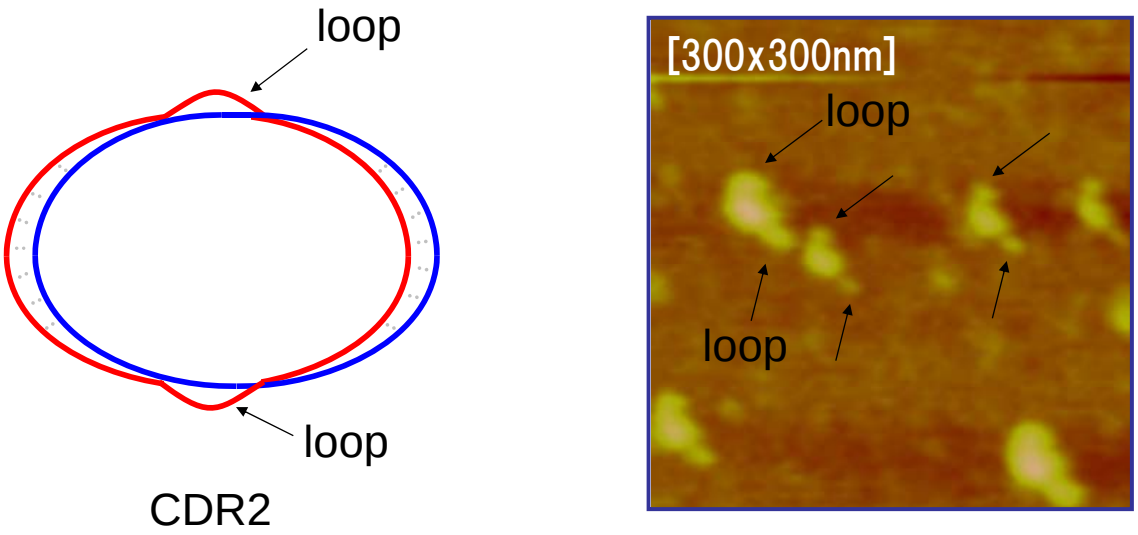


***Figure S4.*** Atomic force microscopy (AFM) showing CDR2 at 300 nm × 300 nm.

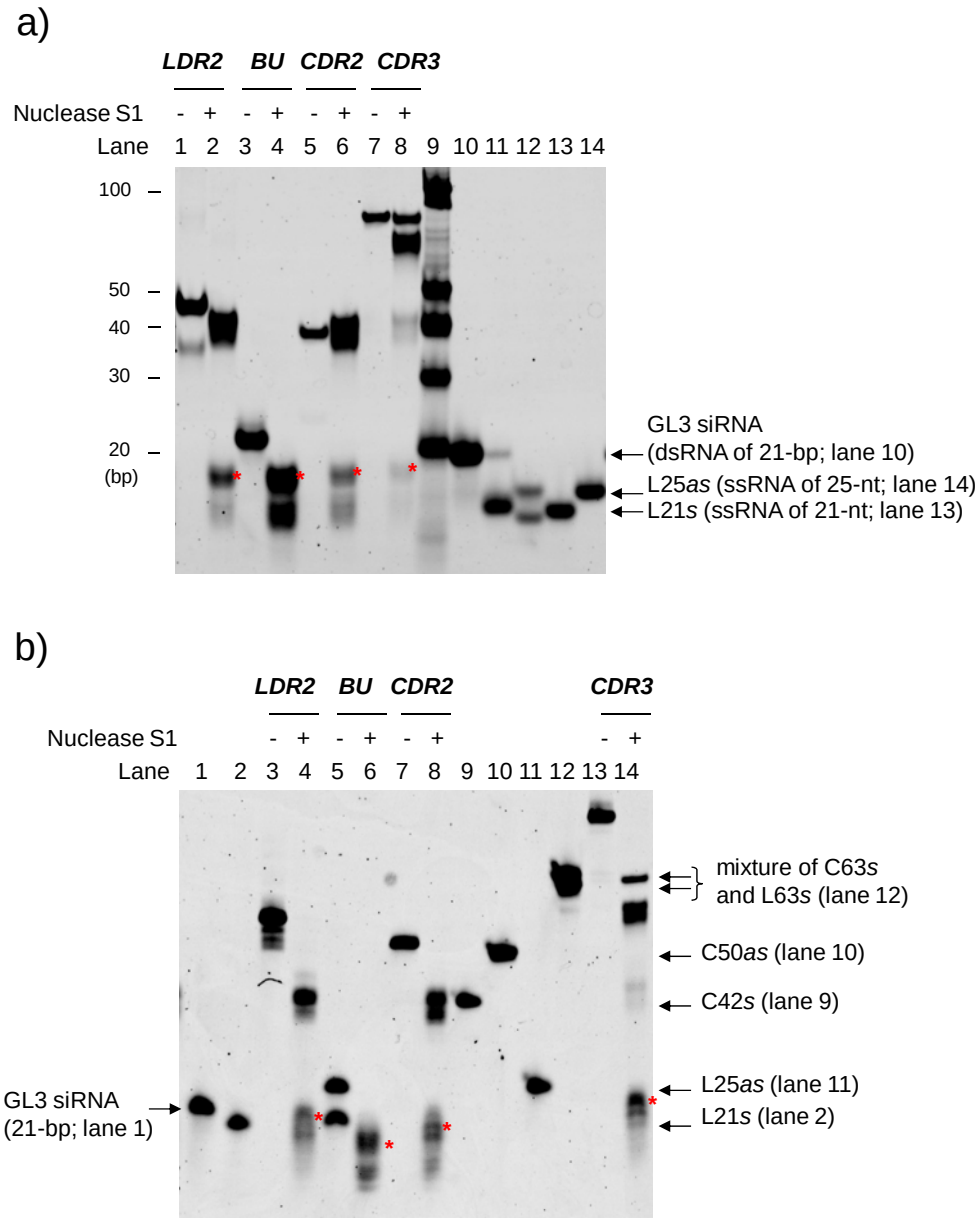


***Figure S5*** Nuclease S1 (S1) digestion of CDRs. (a) non-denaturing PAGE (10%) analysis of the reaction. Gel was visualized by SYBR Green I staining. Lane 1, LDR2; lane 2, LDR2 + S1 ; lane 3, BU ; lane 4, BU + S1; lane 5, CDR2; lane 6, CDR2 + S1; lane 7, CDR3; lane 8, CDR3 + S1; lane 9, dsRNA size marker; lane 10, GL3 siRNA; lane 11, antisense strand of GL3 siRNA; lane 12, sense strand of GL3 siRNA; lane 13, L21*s*; lane 14, L25*as*. (b) denaturing PAGE analysis (10%; 7.5 M urea, 25% formamide) of the reaction. Gel was visualized by SYBR Green II staining. Lane 1, GL3 siRNA; lane 2, L21*s*; lane 3, LDR2; lane 4, LDR2 + S1; lane 5, BU; lane 6, BU + S1; lane 7, CDR2; lane 8, CDR2 + S1; lane 9, C42*s*; lane 10, C50*as*,; lane 11, L25*as*; lane 12, mixture of C63*s* and L63*s*; lane 13, CDR3; lane 14, CDR3 + S1.

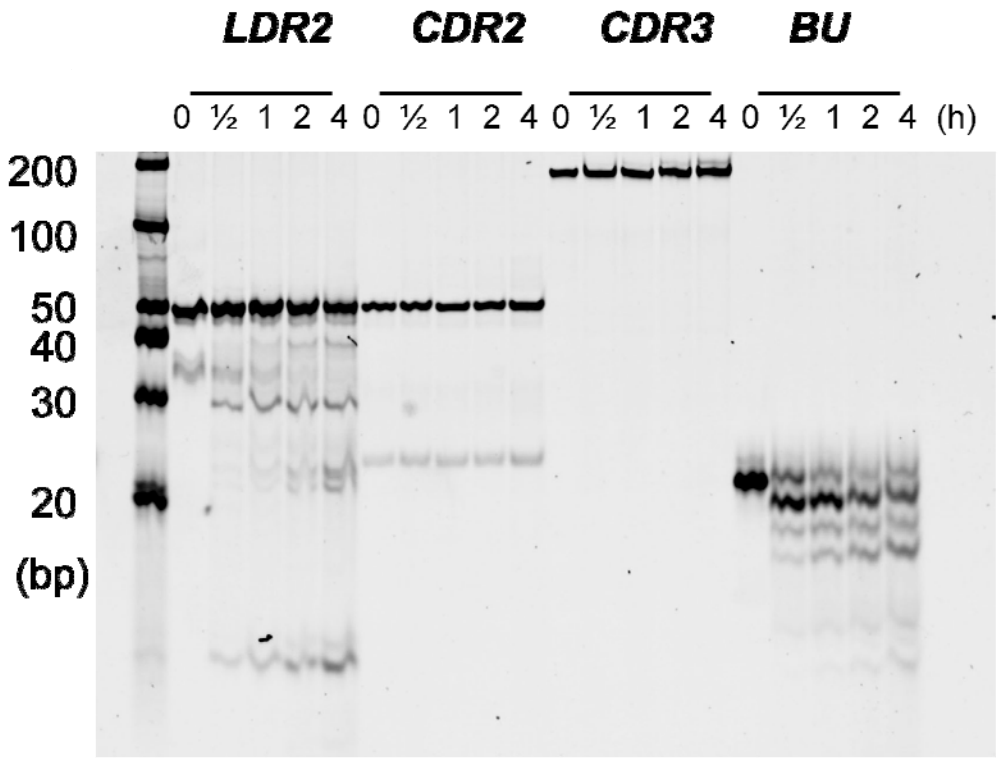


***Figure S6.*** Dicer cleavage reaction of the structured RNAs. RNAs were incubated with Dicer at 37 °C and aliquots were taken after 0.5, 1, 2, and 4 h. The reaction mixtures were analyzed by 15% non-denaturing PAGE, and visualized with SYBR Green I staining.

## Experimental details

### *Synthesis of RNA oligonucleotides*

RNA oligonucleotides were synthesized using 2′-*O*-TOM protected β-cyanoethyl phosphoramidites (Glen Research, USA) on a DNA/RNA synthesizer H-8-SE (Gene World, Japan). 5′-Phosphorylation was performed on the synthesizer using Chemical Phosphorylation Reagent (Glen Research). RNA oligonucleotides were deprotected according to the procedure provided by the manufacturer and were purified by 15% denaturing polyacrylamide gel electrophoresis (PAGE) containing 7.5 M urea, isolated by the crush and soak method. Throughout this study, the ratio of acrylamide to bisacrylamide was 19: 1 (wt/wt). PAGE was performed in TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3). Desalted RNAs were precipitated with sodium acetate (pH 5.2) and 2-propanol. RNA sequences listed in Table 1 except L63*s*/C63*s* were characterized by MALDI-TOF MS spectrometry at the Support Unit for Bio-material Analysis in RIKEN BSI Research Resource Center. siRNA targeting GL3 luciferase [GL3 siRNA, 5′ r(CUUACGCUGAGUACUUCGAUU) 3′/ 5′ r(UCGAAGUACUCAGCGUAAGUU) 3′)] corresponded to the coding region nucleotides 153 – 173 relative to the first nucleotide of the start codon.[1] All structured RNAs targeted the same region.

### *Enzymatic synthesis of circular dsRNAs (CDRs) using T4 RNA ligase.*

At first, single-stranded circular RNAs (C42*s* and C63*s*) were synthesized by ligating its liner starting strand(s) (Figure 2).

Final composition of the reaction mixture to dimerize and circularize L21*s* to form C42*s* was as follows; 1 μM L21*s*, 0.05 units/μL T4 RNA ligase (Takara Bio, Japan), 25% PEG6000 (Merck Chemicals, Germany), 0.006% bovine serum albumin (BSA), 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP. The reaction mixture was incubated at room temperature overnight. After extraction with chloroform, the RNA was precipitated by the addition of sodium acetate (pH 5.2) and 2-propanol. A ligated product C42*s* was purified by preparative (1 mm thick) denaturing PAGE (10% polyacrylamide, 25% formamide, 7.5 M urea). Bands were visualized by UV shadowing, and crushed and extracted with 10 mM EDTA (pH 8.0). The eluate was desalted using a Microcon Ultracel YM-3 cartridge (Millipore, USA). The RNA was then precipitated with sodium acetate (pH 5.2) and 2-propanol.

L63*s* was circularized to C63*s* in the following mixture by incubating at room temperature overnight; 0.25 μM L63*s*, 0.03 units/μL T4 RNA ligase, 25% PEG6000, 0.006% BSA, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP. After extraction with chloroform, the RNA was recovered from the mixture by precipitation. C63*s* was used without further

purification, though its quantitative formation was confirmed by non-denaturing PAGE analysis (10% PAGE) (Figure S1, c).

To synthesize circular double-stranded RNAs, multiple ssRNA molecules were ligated on its circular ssRNA template. ssRNA L25*as* (2.5 – 6 μM) was at first annealed to its complementary circular ssRNA (1 μM; C42*s* for CDR2, C63*s* for CDR3). Final composition of the reaction mixture except RNAs was as follows; 25% PEG6000, 0.006% BSA, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 0.1 – 0.2 units/μL T4 RNA ligase. The mixture was incubated at room temperature overnight. After extraction with chloroform, the RNA was precipitated by the addition of sodium acetate (pH 5.2) and 2-propanol. Products were purified by the same procedure described above to synthesize C42*s* by preparative denaturing PAGE. Isolation yield of circular double-stranded RNAs were as follows; CDR2, 28%: CDR3, 6%: which were calculated based on its circular ssRNAs.

Liner double-stranded RNA LDR2 was synthesized in the same manner described above using L42*s* (1 μM) and L25*as* (2.5 μM), which was isolated in 8% yield.

***Atomic force microscopy (AFM) of structured RNA molecules.***

A drop of the RNA sample (200 pM RNA in 10 mM Tris-HCl, pH 7.2, 12.5 mM $MgCl_2$, 1 mM EDTA) was spotted on the mica, which was cleaved freshly and pretreated with 3 μL of 1 mM $NiCl_2$. After 30 s, the unbound RNA was removed by rinsing with deionized water. After being dried in a silica-gel desiccator for one day, AFM images were obtained under ambient conditions with Nanoscope IIIa (Veeco Instruments Inc., USA) by tapping mode with SSS-NCH tip (NanoWorld AG, Switzerland).

***Measurements of RNA interference activity of the structured RNAs.***

The concentration of all structured RNAs was adjusted by dividing it by the number of its siRNA motif in this assay. All RNAs used were annealed beforehand in 1× annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) by heating at 90 °C for 3 min, then cooled slowly to room temperature. The solutions were further incubated at 4 °C overnight.

NIH 3T3 cells (RIKEN Cell Bank, Japan) were grown at 37 °C under 5% $CO_2$ in minimum essential medium eagle (DMEM; Wako, Japan) supplemented with 10% fetal bovine serum (FBS; Biological Industries Ltd., Israel), 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were regularly passaged to maintain exponential growth. One day before transfection at 50% confluency, cells were plated in 96-well plates ($5 \times 10^3$ cells/100 μL per well). Cotransfection of reporter plasmids and RNAs was carried out with GeneSilencer (Genlantis, USA) as described by the manufacturer for adherent cell lines. Just before cotransfection, the

culture medium was replaced with 56 μL of antibiotics-free DMEM without serum, and 0.02 μg of pGL3-Control (Promega, USA), 0.02 μg of pRL-TK (Promega), and RNA (10 pmol equivalent) formulated into liposomes (44 μL) were added to each well. Four hours after the transfection, 100 μL of 20% FBS in DMEM was added to each well. Two days (48 h) after the transfection, luciferase expression was monitored with the Dual-Luciferase Reporter Assay System (Promega) on a Wallac 1420 ARVO SX multilabel counter (PerkinElmer, USA).

***Nuclease S1 digestion of the structured RNAs***

RNAs were incubated at 2 μM equivalent concentration with Nuclease S1 (2.4 units/μL; Takara Bio) in 30 mM sodium acetate (pH 4.6), 280 mM NaCl, 1 mM $ZnSO_4$ at 25 °C for 2h. The reaction was stopped by adding EDTA to a final concentration of 30 mM. They were analyzed by 10% non-denaturing PAGE and 10% denaturing PAGE (7.5 M urea, 25% formamide). siRNA Ladder Marker (Takara Bio, Japan) was used as the size marker for dsRNA in the non-denaturing PAGE. The gel was stained with SYBR Green I (for non-denaturing; Roche, Switzerland), or SYBR Green II (for denaturing; Lonza, Switzerland) visualized by scanning on a BioRad Molecular Imager FX (BioRad, USA).

***Dicer cleavage reaction of the structured RNAs***

RNAs (0.91 μM equivalent concentration) were mixed with Dicer enzyme (0.091 units/μL; Recombinant Human Turbo Dicer Enzyme Kit from Genlantis, USA) in the buffer system supplied. The mixtures were incubated at 37 °C and aliquots (2.2 μL) were taken from the mixture after 0.5, 1, 2 and 4 h. They were analyzed by 15% non-denaturing PAGE. siRNA Ladder Marker (Takara Bio, Japan) was used as the size marker for dsRNA in the non-denaturing PAGE. The gel was stained with SYBR Green I (for non-denaturing; Roche, Switzerland), visualized by scanning on a BioRad Molecular Imager FX (BioRad, USA).

**Reference**

[1] S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, *Nature* **2001**, *411*, 494.