RNA ligation of very small pseudo nick structures by T4 RNA ligase 2, leading to efficient production of versatile RNA rings

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Table S1 The RNA sequences used in this study.

Name	Sequence（5′ → 3′）	Length（nt）
L-22a	ACUGCCCCAGUUAGCCCCGCUA	22
L-22b	CCCGCCCAAAUUAGCCCCGCUA	22
L-22c	ACUGCCCCAGUUAAACCCGCCC	22
L-22d	CCCGCCCAAAUUAAACCCGACC	22
L-8	CCUAAUCA	8
L-21	CCUAAUCACGGUCAAGGACCG	21
L-24	ACUGCCCCAGUUUUAGCCCCGCUA	24
L-23a	ACUGCCCCAGUUAGCCCCGCUAU	23
L-18	GGCCCCCGAGCCCCGCUC	18
L-15	GACCUCGGCCCCGCC	15
L-12	GAGUCCGCCCCG	12
L-23b	CUGAGUUGUGUUCUGCUGUUGUU	23
L-23c	UGCUGUUGUUCUGAGUUGUGUUC	23
L-31a	CCCUUAUAAUUUCCUCCUCCAUAGUUUCCUU	31
L-31b	UCCUUCCCUUAUAAUUUCCUCCUCCAUAGUU	31

Table S2 The DNA sequences used in this study.

Name	Sequence（5′→3′）	Length（nt）
DNA21	CGGTCAAGGACCGTGATTAGG	21
DNA19	CGGTCAAGGACCGTGATTA	19
DNA17	CGGTCAAGGACCGTGAT	17
DNA15	CGGTCAAGGACCGTG	15
DNA13	CGGTCAAGGACCG	13
DNA10a-8	TGATTAGGAA	10
DNA10a-6	TGATTAAAAA	10
DNA10a-4	TGATATAAAA	10
DNA10a-2	TGCAATAAAA	10
DNA10a-0	ACCAATAAAA	10
DNA8a	GCTAATTT	8
DNA10b	GGCTAATTTG	10
DNA12	GGGCTAATTTGG	12
DNA22	GGTCGGGTTTAATTTGGGCGGG	22
DNA8b	TGATTAGG	8
DNA6	TGATTA	6
DNA5	TGATT	5

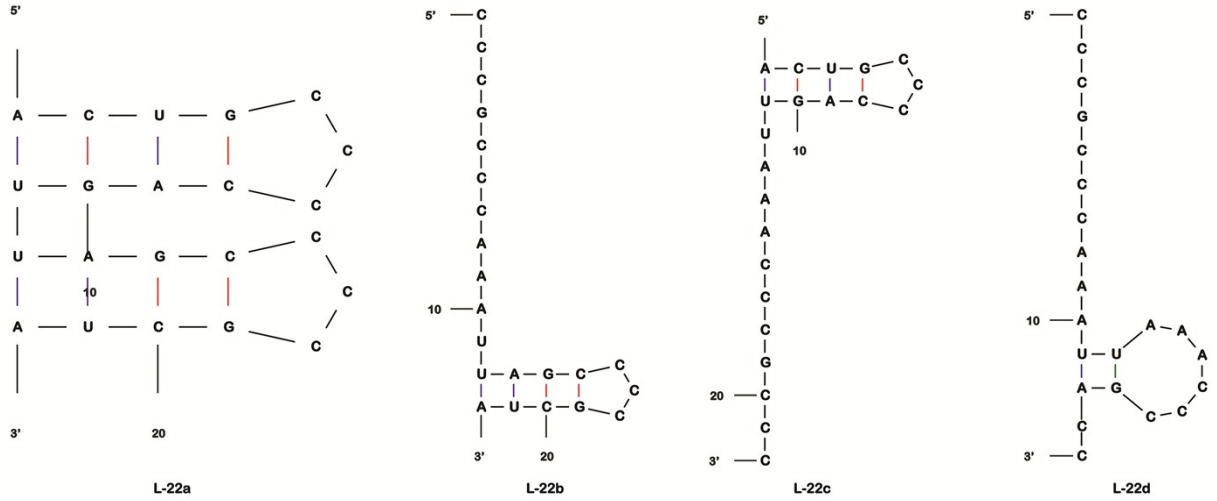

Fig. S1 The secondary structures of RNAs were simulated by Mfold software ([Mg^{2+}] = 2 mM, 25°C).

The mfold structures of L-22a, L-22b, L-22c and L-22d are shown.

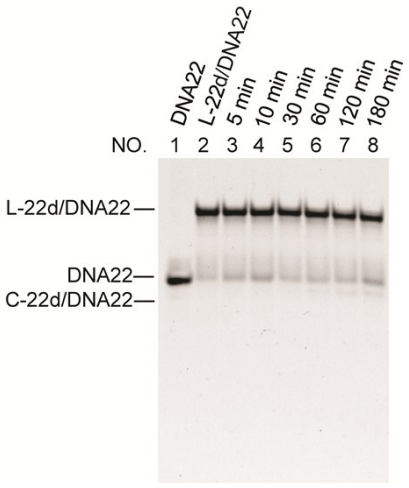

Fig. S2 Non-denaturing PAGE (15%) for intramolecular ligation of L-22d without any secondary structure.

Since the single-stranded L-22d RNA cannot be clearly stained by SYBR Gold, its complementary DNA sequence was added to assist the dyeing. Accordingly, the bands shown here are duplexes. Lane 1, only DNA22; lane 2, L-22d + DNA22; lane 3-8: L-22d treated with Rnl2 for 5 min, 10 min, 30 min, 60 min, 120 min and 180 min, respectively (then, DNA22 as the complementary DNA of L-22d was added). [RNA] = 1 μM, [DNA] = 1 μM and [Rnl2] = 0.4 U/μL in 1×buffer at 25°C.

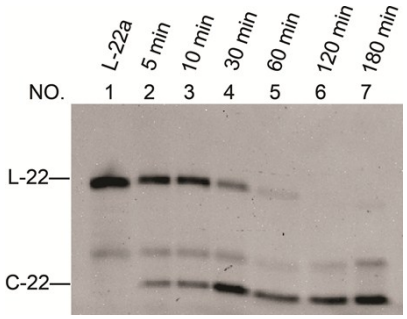

Fig. S3 Denaturing PAGE (12%) analysis for intramolecular cyclization of L-22a by Rnl2.

Lane 1, only L-22a; lane 2-7: L-22a treated with Rnl2 for 5 min, 10 min, 30 min, 60 min, 120 min and 180 min, respectively. [RNA] = 1 μM and [Rnl2] = 0.04 U/μL in 1×buffer at 25°C.

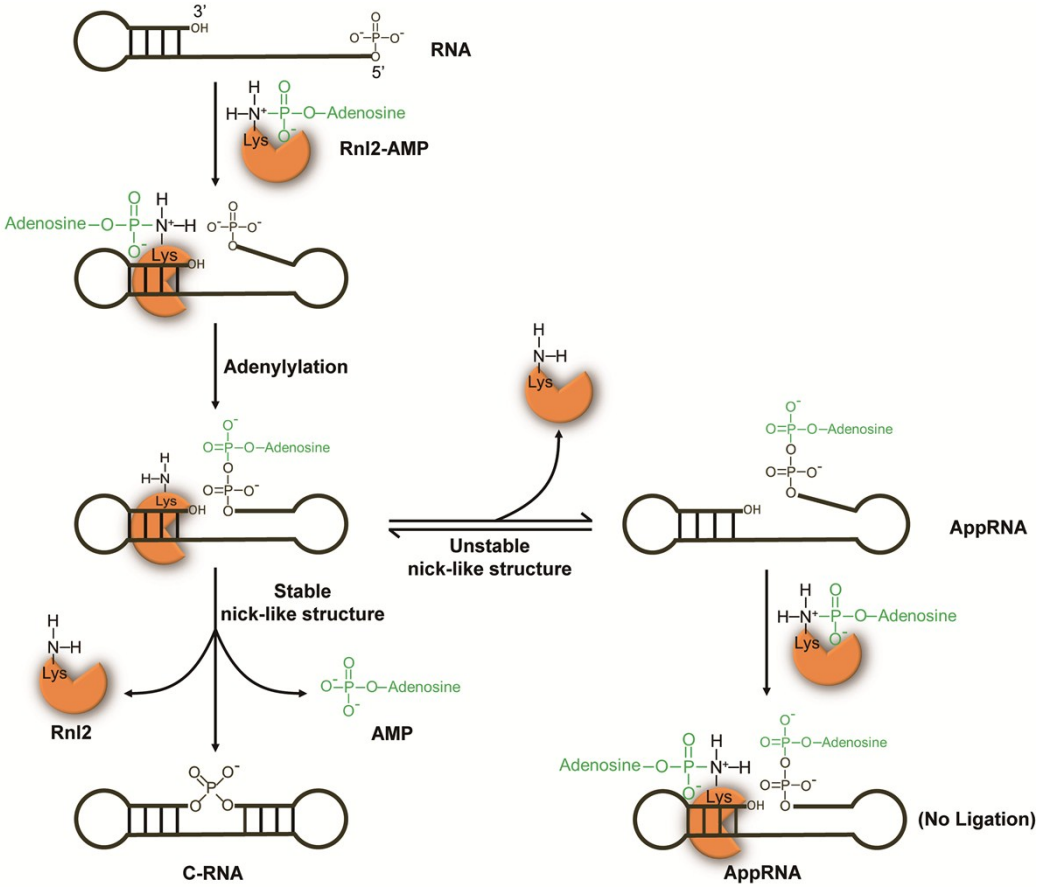

Fig. S4 Schematic diagram of RNA ligation by forming pseudo nick structures.

At first, the adenylated Rnl2 binds to the duplex structure at 3′-end, and then the 5′-phosphate is adenylated. If the 5′-end can form a duplex structure which is stable enough, the ligation happens efficiently. Otherwise, the ligation hardly happens.

The RNA involving double-stranded structure (or pseudo duplex) at 3′-end is required to be bound by adenylated Rnl2 (Rnl2-AMP). Only in this case, the 5′-phosphoate can be adenylated. In other words, duplex structure at 3′-end is essential for 5′-adenylation. If the stable nick-like structure exists, the ligation occurs efficiently. If non-stable nick-like structure forms, the ligation hardly happens, and only the RNA-adenylate intermediate (AppRNA) forms in the case that nick-like structure is unstable enough.

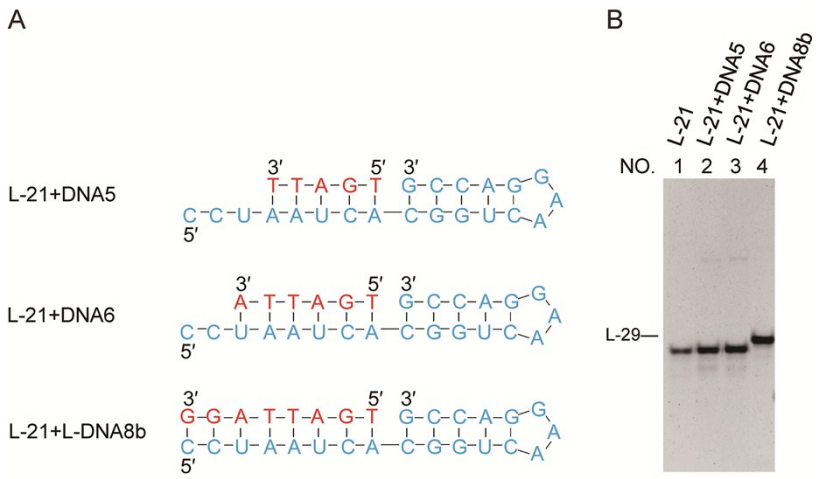

Fig. S5 Dependence of the ligation efficiency on the length of duplex part (DNA/RNA heteroduplex) in the 5′-phosphate side of joining site (as well as in Fig. 3).

(A) The sequences of RNA (blue) and DNA (red). (B) Denaturing PAGE (12%) analysis for the ligation at 25°C for 12 h. Lane 1, L-21 only. Lane 2, ligation of L-21 with DNA5. Lane 3, ligation of L-21 with DNA6. Lane 4, ligation of L-21 and DNA8b. [RNA] = 1 μM, [DNA] = 2 μM and [Rnl2] = 0.4 U/μL in 1×buffer.

Here, all the ligations are believed to occurred, although we don't know the exact yields. The intermolecular ligation products of ssRNA (L-21) and ssDNA (DNA5 or DNA6) showed similar mobility with the substrate of L-21 on the PAGE gel (compare lane 2 and lane 3 with lane 1), because these products involve very stable hairpin structures.

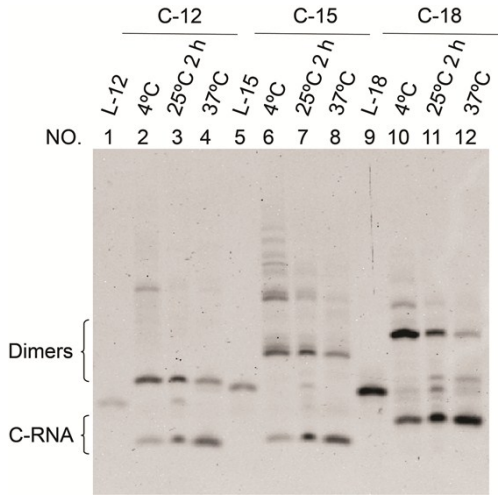

Fig. S6 Effects of temperature on cyclization of L-12, L-15 and L-18.

Lanes 1, 5 and 9, L-12, L-15 and L-18 only. Lanes 2, 6 and 10, L-12, L-15 and L-18 treated with Rnl2 at 4°C for 12 h. Lanes 3, 7 and 11, L-12, L-15 and L-18 treated with Rnl2 at 25°C for 2 h. Lanes 4, 8 and12, L-12, L-15 and L-18 treated with Rnl2 at 37°C for 12 h. [RNA] = 1 μM and [Rnl2] = 0.4 U/μL in 1× buffer. 12% denaturing PAGE was used.

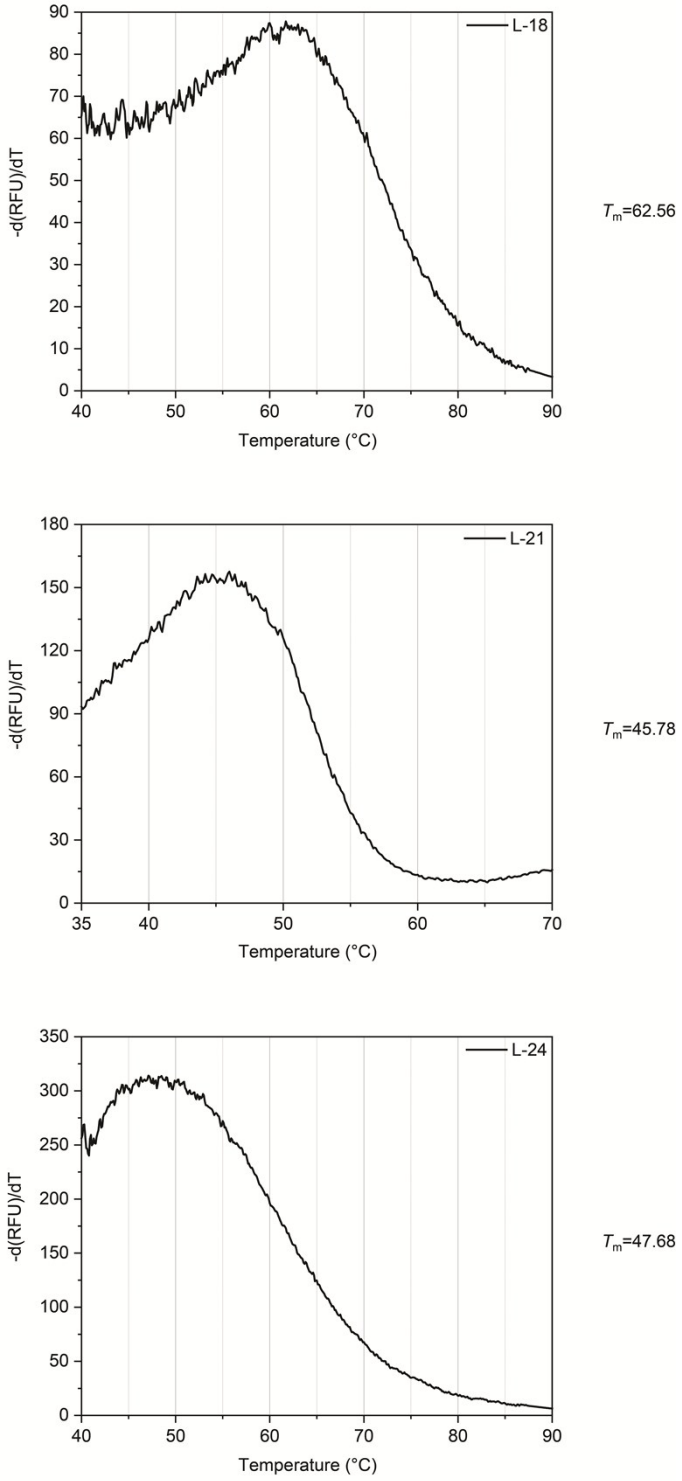

Fig. S7 High resolution melting curve analysis of L-18, L-21 and L-24.

–d(RFU)/dT: the rate of change of the relative fluorescence units (RFU) with time (T). The RNAs were measured at 1 μM in 10 μL solution containing 1×EvaGreen and 1×Rnl2 buffer.

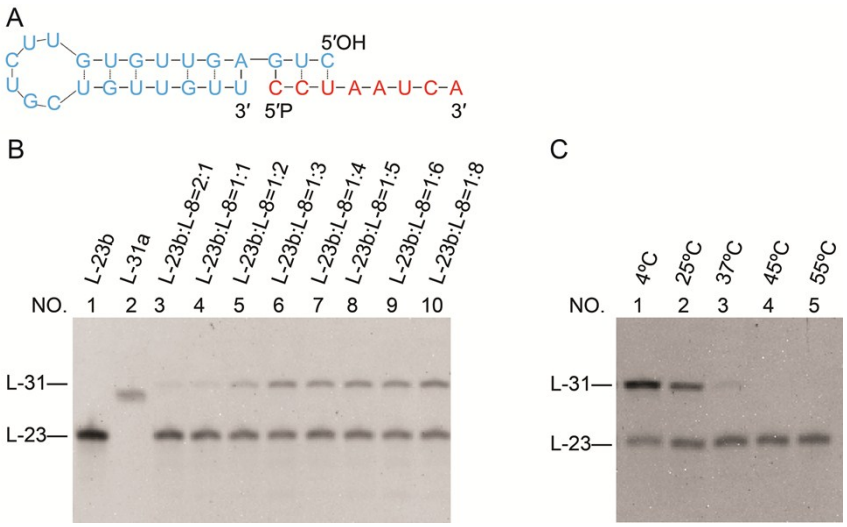

Fig. S8 Concentration effects on the ligation of L-23b and L-8 involving highly unstable stems.

(A) Schematic diagram of connection between L-23b and L-8. Only L-8 was 5′-terminal phosphorylated. (B) Denaturing PAGE (12%) for the ligation product. [L-8] = 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 μM in lanes 3-10, respectively ([L-23b] = 0.5 μM at 25°C for 6 h). L-8 cannot be observed on the gel. (C) Temperature dependence of the ligation of L-23b and L-8. [L-23b] = 0.5 μM, [L-8] = 4.0 μM.

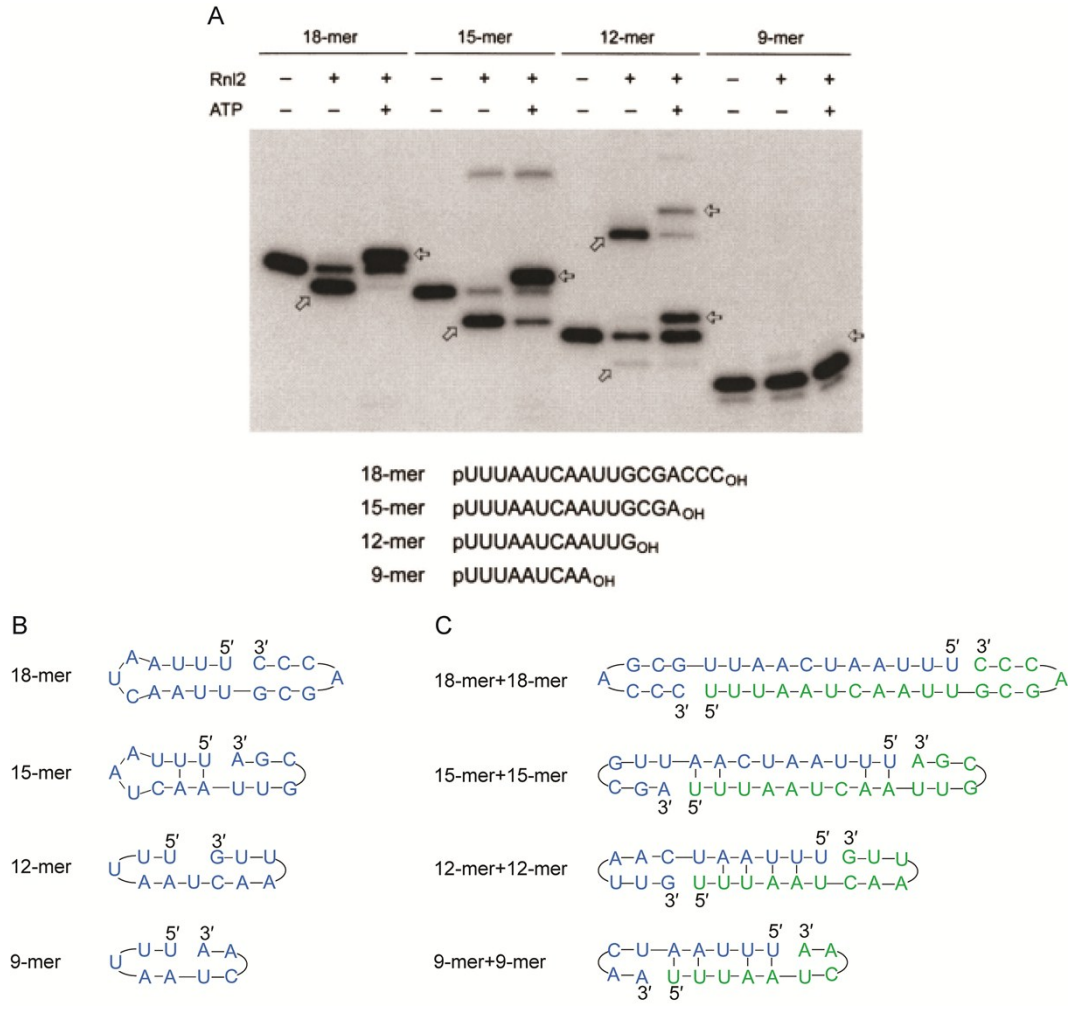

Fig. S9 Analysis of the results in the literature in terms of the "nick-like structure mechanism".

(A) PAGE analysis of cyclization of 9-mer, 12-mer, 15-mer and 18-mer (from Yin S, Ho C K, Miller E S, Shuan S., Characterization of bacteriophage KVP40 and T4 RNA ligase 2. Virology, 2004, 319(1):141). In (B) and (C), the nick-like structures we propose for intramolecular and intermolecular cyclization are presented, respectively.

This reference showed that 1) 18-mer RNA only forms monomeric circle; 2) 15-mer RNA could be circularized to form monomeric circle, but dimeric product was observed; 3) Ligation of 12-mer RNA almost gave only dimeric product; 4) Neither monomeric nor dimeric products were obtained for the 9-mer RNA substrate. These results are satisfactorily interpreted in terms of "nick-like structure mechanism".

As shown in (B), 12-mer was not ligated to a circle because no nick-like structure could be formed. Both 15-mer and 18-mer formed nick-like structure so that they could be circularized to monomeric ring. 15-mer showed higher yield than 18-mer, because of the formation of more stable nick-like structure. The stability of intermolecular secondary structures in (C) was in the following order: 12-mer > 15-mer > 18-mer, which was in accordance with their efficiency for the formation of dimers. For 9-mer, no ligation occurred because it could not form any effective secondary structure. Alternatively, the nick-like structure of 9-mer could be too short for Rnl2 to bind for ligation.