Supplementary Information

Efficient circular RNA synthesis through Gap-DNA splint-mediated ligation

Hyunji Kim[a,1], Dajeong Kim[a,1], Sunghyun Moon[a], and Jong Bum Lee[a,]*

[a.] Department of Chemical Engineering, University of Seoul, 163 Seoulsiripdaero, Dongdaemun-gu, Seoul 02504, Republic of Korea

[1.] These authors contributed to the work equally.

[*]Corresponding author: jblee@uos.ac.kr

Experimental Section

Synthesis of the linear RNAs by in vitro transcription 179 nt of single-stranded DNA templates and an 18 nt of DNA oligomer (10 μM) containing the T7 promotor sequence were annealed by heating at 95°C for 2 minutes, followed by gradual cooling to 25°C for more than 1 hour using a thermal cycler (Bio-Rad) to make double-stranded DNA (dsDNA) template. All oligo DNAs were purchased from Integrated DNA Technologies (Coralville, Iowa, USA). 153 nt of linear RNA was synthesized from the dsDNA template by in vitro transcription using the EZ T7 High Yield In Vitro transcription kit (Enzynomics, Daejeon, Repulic of Korea), according to the manufacturer's protocol. 5'-monophosphorylated in vitro transcripts were obtained by adding an excess amount of GMP (100 mM, Sigma Aldrich) to GTP (25 mM) in the reaction mixture. After the reaction, the template DNA was removed by DNase I (Epicentre) treatment, and the RNA was purified by ethanol precipitation.

Circularization of linear RNA using T4 RNA ligase 2 The transcribed linear RNA was ligated using T4 RNA ligase 2 (New England Biolabs) on a 22 nt DNA sequence (DNA splint) as a template. A typical reaction system (40 μL) was composed of annealed linear RNA (2 μM) and DNA splint (2 μM), T4 RNA ligase 2 (4 U μL^{-1}) in 1X T4 RNA ligase 2 buffer (50 mM Tris-HCl (pH7.5), 2 mM MgCl2, 1 mM DTT, and 0.4 mM ATP) at 15℃ or 37℃ for 2 hours.

Gap-DNA splint-mediated circularization The linear RNA was annealed using a matched or mismatched DNA splint that includes a 1 nt gap, and the corresponding rNTP was added at a concentration of 1~100 μM during ligation step. After ligation, the DNA splint was removed by DNase I (Epicentre) treatment. When necessary, 16 or 32 units of T4 RNA ligase 2 were used.

Circularity check of the RNA using RNase R and RNase I The RNA (0.04 μg/μL) was incubated with RNase R (exonuclease) or RNase I (endonuclease) at 37℃ for 10 min. After the enzyme reaction, samples were loaded onto 8% denaturing UREA PAGE gel, and electrophoresis was carried out at 180 V for 40 min. The gel was then stained with GelRed. The images were then analyzed with Image J software to compare the circularity efficiency.

Agarose gel electrophoresis The RNA (0.04 μg/μL) was incubated with RNase R (exonuclease) at 37℃ for 10 min. After the enzyme reaction, samples were loaded onto 3% agarose gel, and electrophoresis was carried out at 80 V for 100 min. The gel was then stained with GelRed. The images were analyzed with Image J software to compare the circularity efficiency.

Template DNA for Linear RNA transcription (179 nt)	5’ ACC AGT TTG ATG ATG CTA ATC TTC TTG AAC AGC CGC CAG CCG CTC ACG ATG ATC AGT TTT TCA AAG TTG ATT ATA CTG ATG ATC AGT TTT TCA AAG TTG ATT ATA CTG ATG ATC AGT TTT TCA AAG TTG ATT ATA CTG TCC ATG GTG GCT CCC TAT AGT GAG TCG TAT TAG GAT CCG CG 3’
T7 promoter	5’ TAA TAC GAC TCA CTA TAG 3’
DNA Splint (22 nt)	5’ GTC CAT GGT GGC TCC C^{V}ACC AGT 3’
Mismatched Gap-DNA Splint (23 nt)	5’ GTC CAT GGT GGAC TCC C^{V}ACC AGT 3’
ATP Gap-DNA Splint (23 nt)	5’ GTC CAT GGT GGC TCC C^{V}AA CCA GT 3’
TTP Gap-DNA Splint (23 nt)	5’ GTC CAT GGT GGC TCC C^{V}TA CCA GT 3’
GTP Gap-DNA Splint (23 nt)	5’ GTC CAT GGT GGC TCC C^{V}GA CCA GT 3’
CTP Gap-DNA Splint (23 nt)	5’ GTC CAT GGT GGC TCC C^{V}CA CCA GT 3’

Table S1. Sequences of template DNA and DNA Splint for the synthesis of linear RNA and circular RNA. The symbol ^{V} indicates the position of nick after hybridization with ssRNA. Gap-DNA splint and mismatched Gap-DNA splint are 23 nt in length, with the gap base (marked in red) located at different positions. The orange and underlined parts represent the DNA splints and the complementary part to the T7 promoter, respectively.

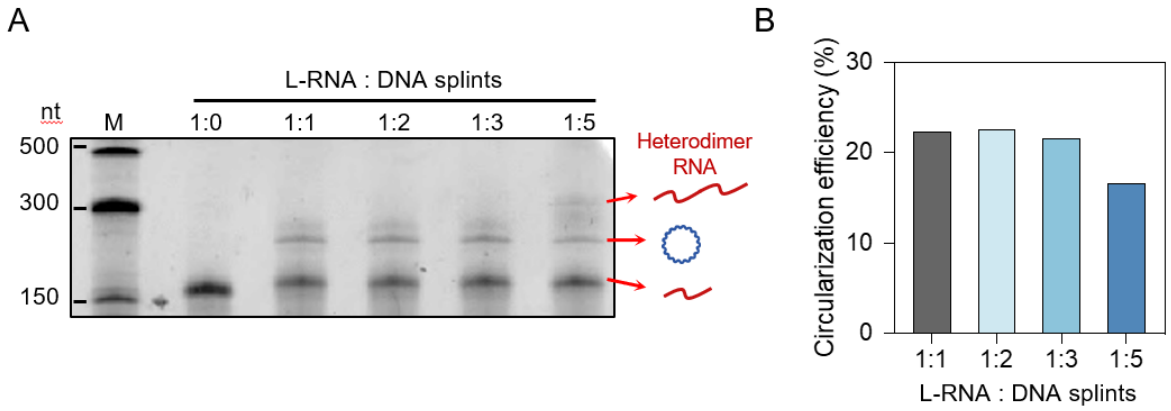

Figure S1. Optimal circularization conditions to prevent heterodimer ligation. (A, B) The electrophoresis result of circular RNA synthesis by adjusting the ratio of linear RNA and splint DNA. As the ratio of splint DNA increases, more multimeric RNA is produced, so heterodimer ligation can be prevented by maintaining the ratio of linear RNA and splint DNA at 1:1. (B) Analysis of circularization efficiency based on the relative band intensity of C-RNA. The gel analysis was analyzed by 8% denaturing UREA PAGE.

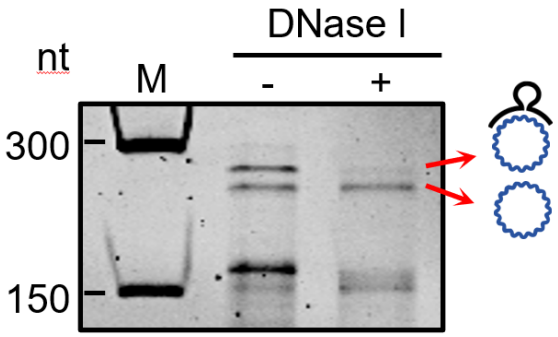

Figure S2. Gel electrophoresis result of naked GS-RNA or GS-RNA treated with DNase 1. The experiment was analyzed by 8% denaturing UREA PAGE.