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Supplemental information

Efficient circular RNA engineering by end-to-end self-targeting and splicing reaction using *Tetrahymena* group I intron ribozyme

Kyung Hyun Lee, Seongcheol Kim, Jaehwi Song, Seung Ryul Han, Ji Hyun Kim, and Seong-Wook Lee

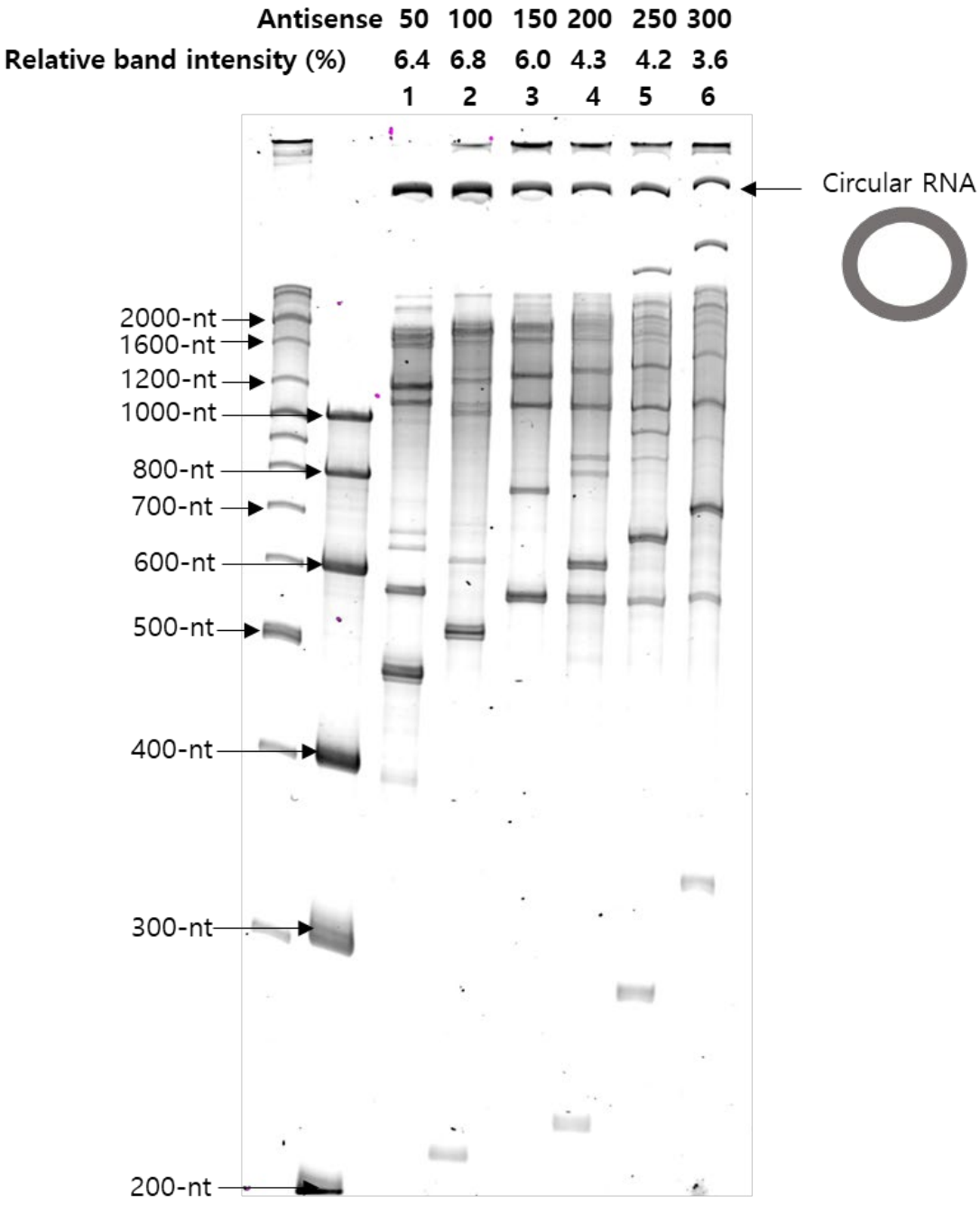

Figure S1. Results of 4% denatured PAGE analysis of IVT samples of self-circularization RNA construct (GOI: EMCV IRES-Gaussia Luciferase) with different sizes of antisense sequence and antisense binding sequence (lane 1: 50-nt, lane 2: 100-nt, lane 3: 150-nt, lane 4: 200-nt, lane 5: 250-nt, lane 6: 300-nt long antisense sequence and antisense binding sequence). Relative band intensity of circRNA is shown as a percentage of whole intensity of each lane.

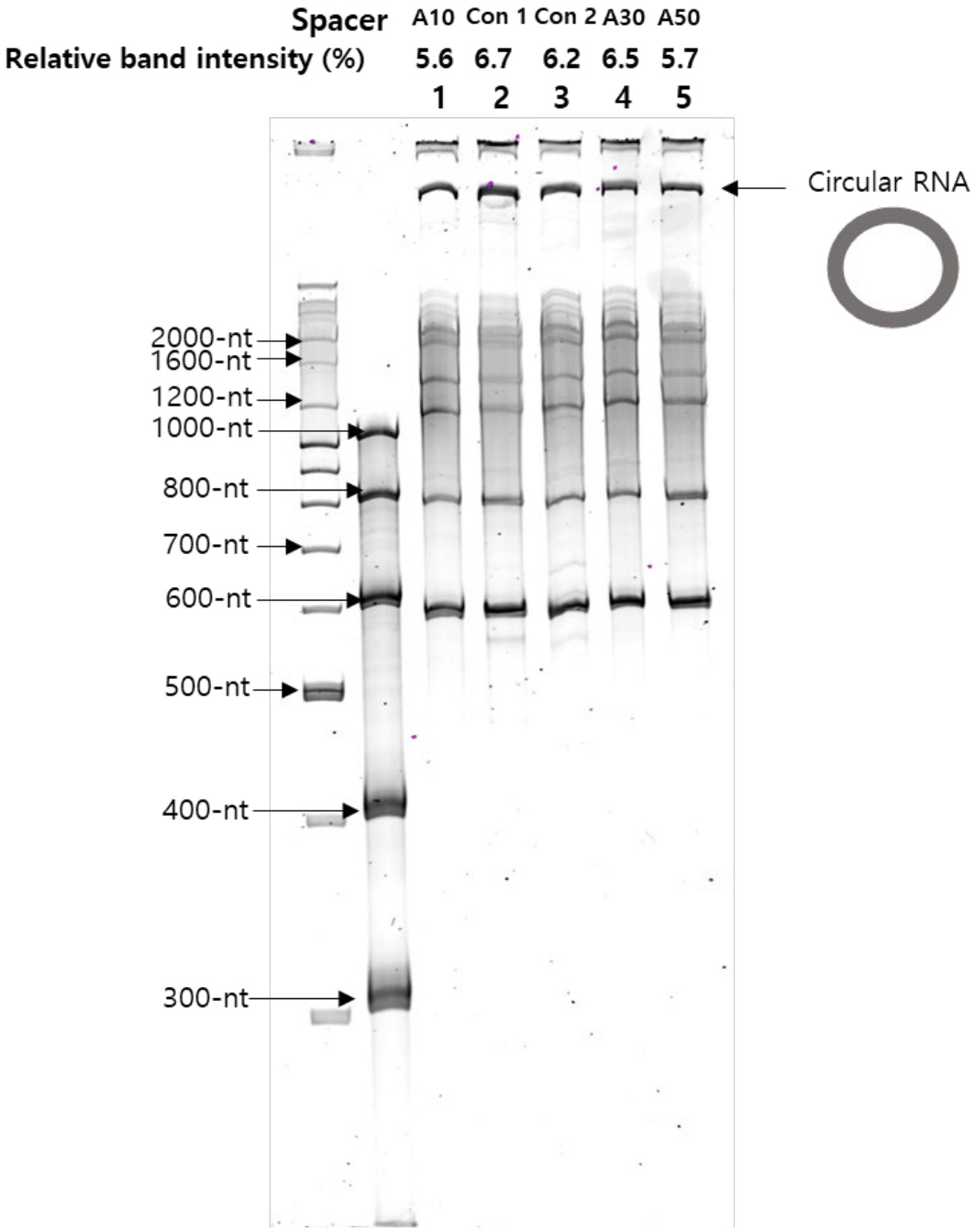

Figure S2. Results of 4% denatured PAGE analysis of IVT samples of self-circularization RNA construct (GOI: EMCV IRES-Gaussia Luciferase) with various spacers (linkers) between group I intron and IRES (lane 1: A10, lane 2: control 1, lane 3: control 2, lane 4: A30, lane 5: A50 spacer). Relative band intensity of circRNA is shown as a percentage of whole intensity of each lane.

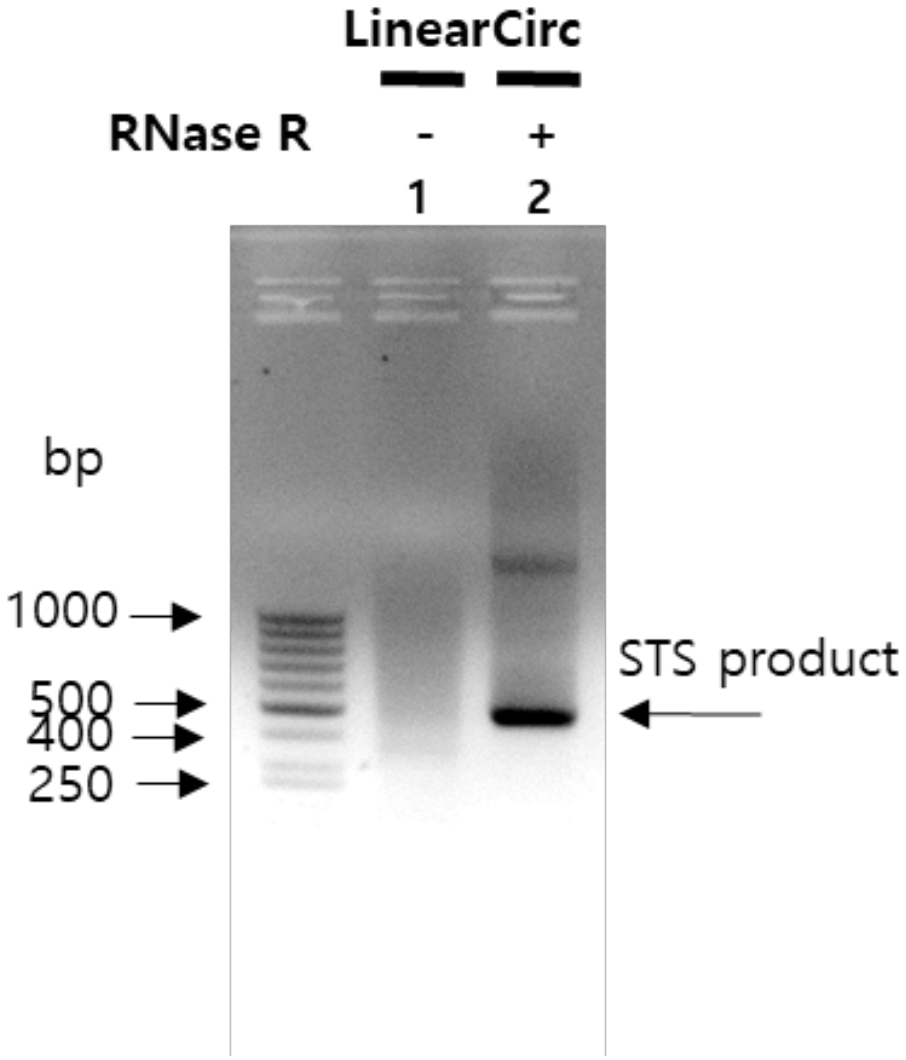

Figure S3. Results of 1.5% agarose gel analysis of RT-PCR products using STS primers listed in Table S3 for circRNA detection for control linear RNA without RNase R digestion (lane 1) and circular RNA IVT samples (P1 construct of Figure 3, GOI: EMCV IRES-Gaussia luciferase) with RNase R digestion (lane 2). Designed STS primers only amplify circular RNA. Arrow indicates the expected circRNA band.

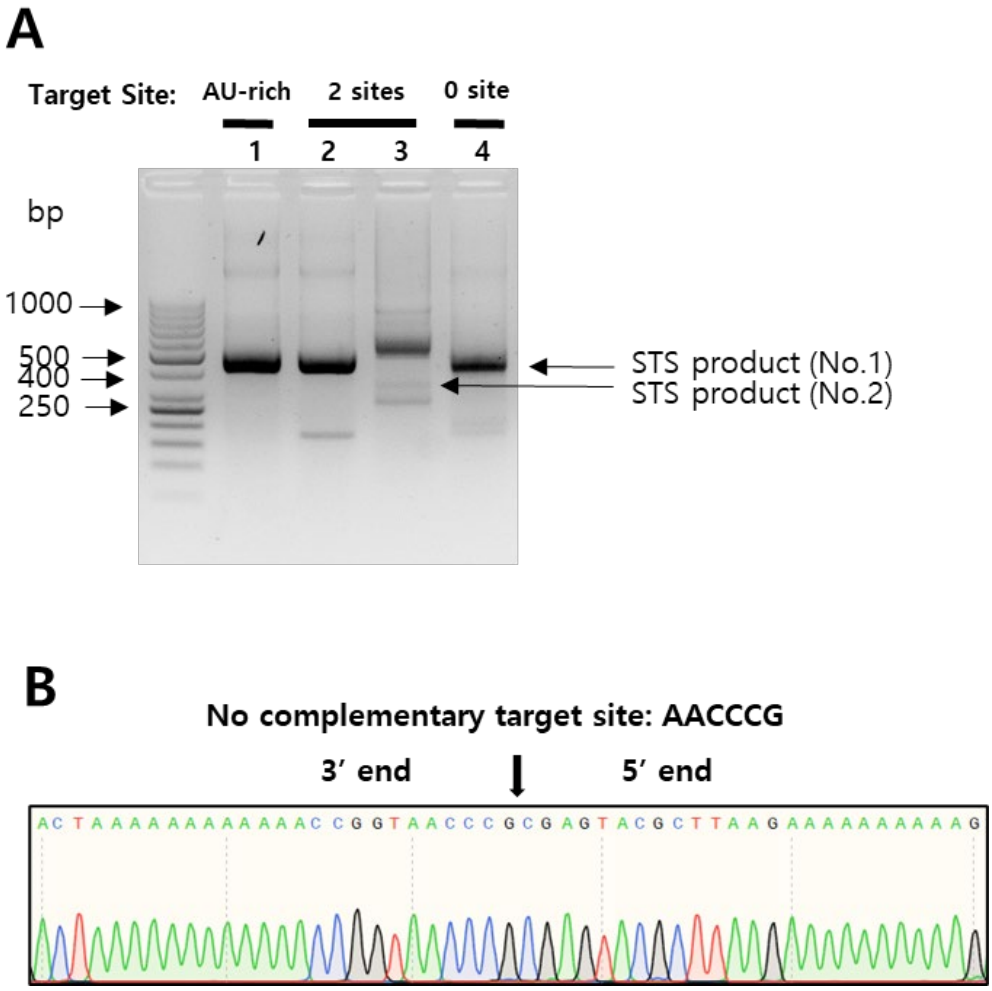

Figure S4. RT-PCR and sequencing analysis of selected P1 constructs of Figure 4 (GOI: EMCV IRES-Gaussia luciferase) with different target sequences. (**A**) Results of 2% agarose gel analysis of RT-PCR products using STS primers for circRNA detection (lane 1: AU-rich target site, lane 2: target No. 1 site present at 3′ end of RNA construct with 2 target sites, lane 3: target No. 2 site present in the middle of GOI for RNA construct with 2 target sites, lane 4: no complementary target site). STS F and R primers were used for lane 1, 2, and 4. STS F2 and R was used for lane 3. Lane 2 and 3 are from same self-circularization RNA, but amplified by different primer sets. (**B**) Sequencing analysis of RT-PCR band from circRNA prepared by P1 construct without complementary target site. Ligation junction region is shown.

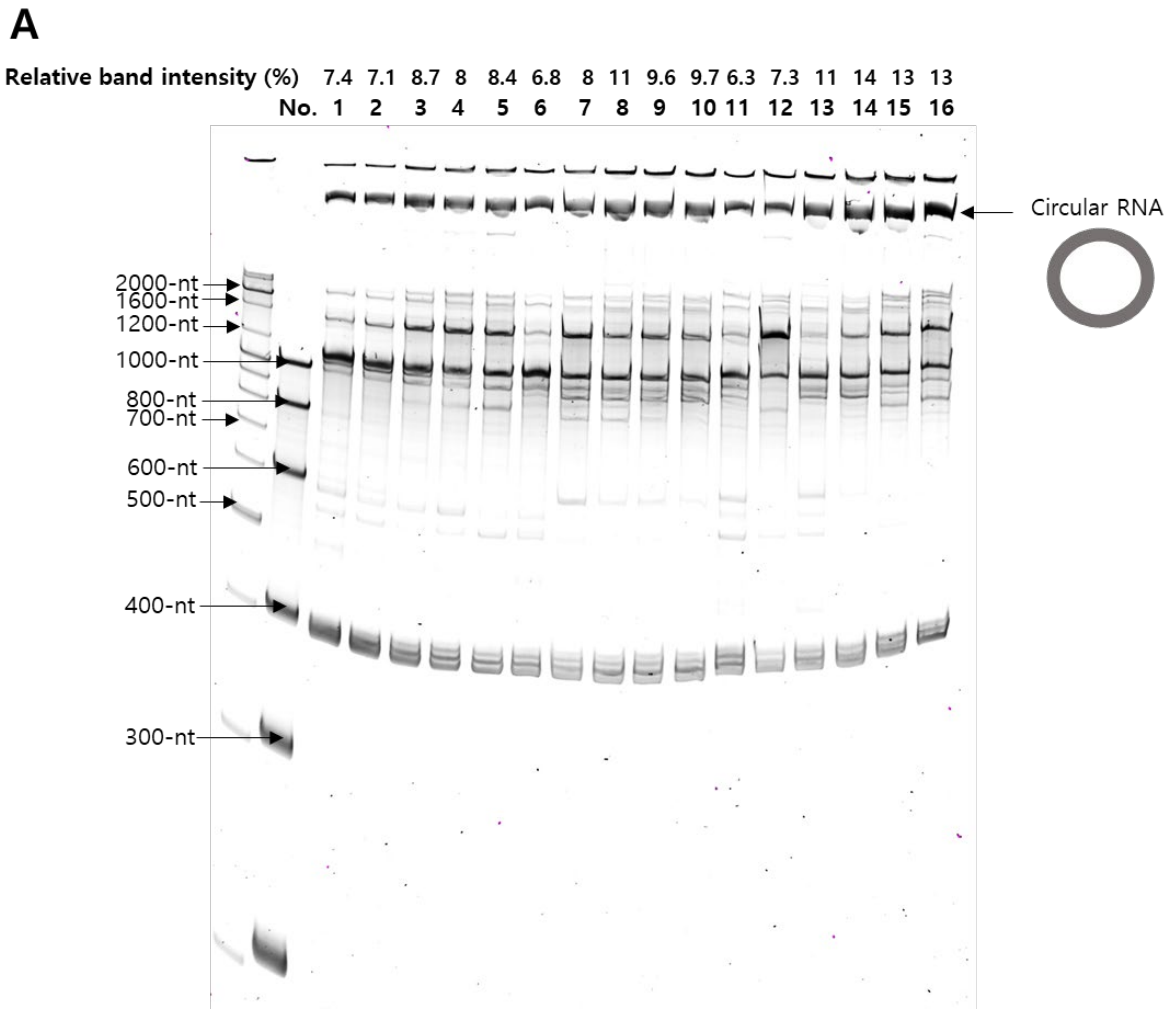

Figure S5. Results of 4% denatured PAGE analysis for IVT samples of self-circularization RNA P1 construct (GOI: EMCV IRES-Gaussia luciferase) with different AU-rich target sequences. (**A**) AU-rich No. 1 to 16 RNA constructs. Target sequence of each No. of AU-rich RNA construct is listed in Table S2. Relative band intensity of circRNA is shown as a percentage of whole intensity of each lane.

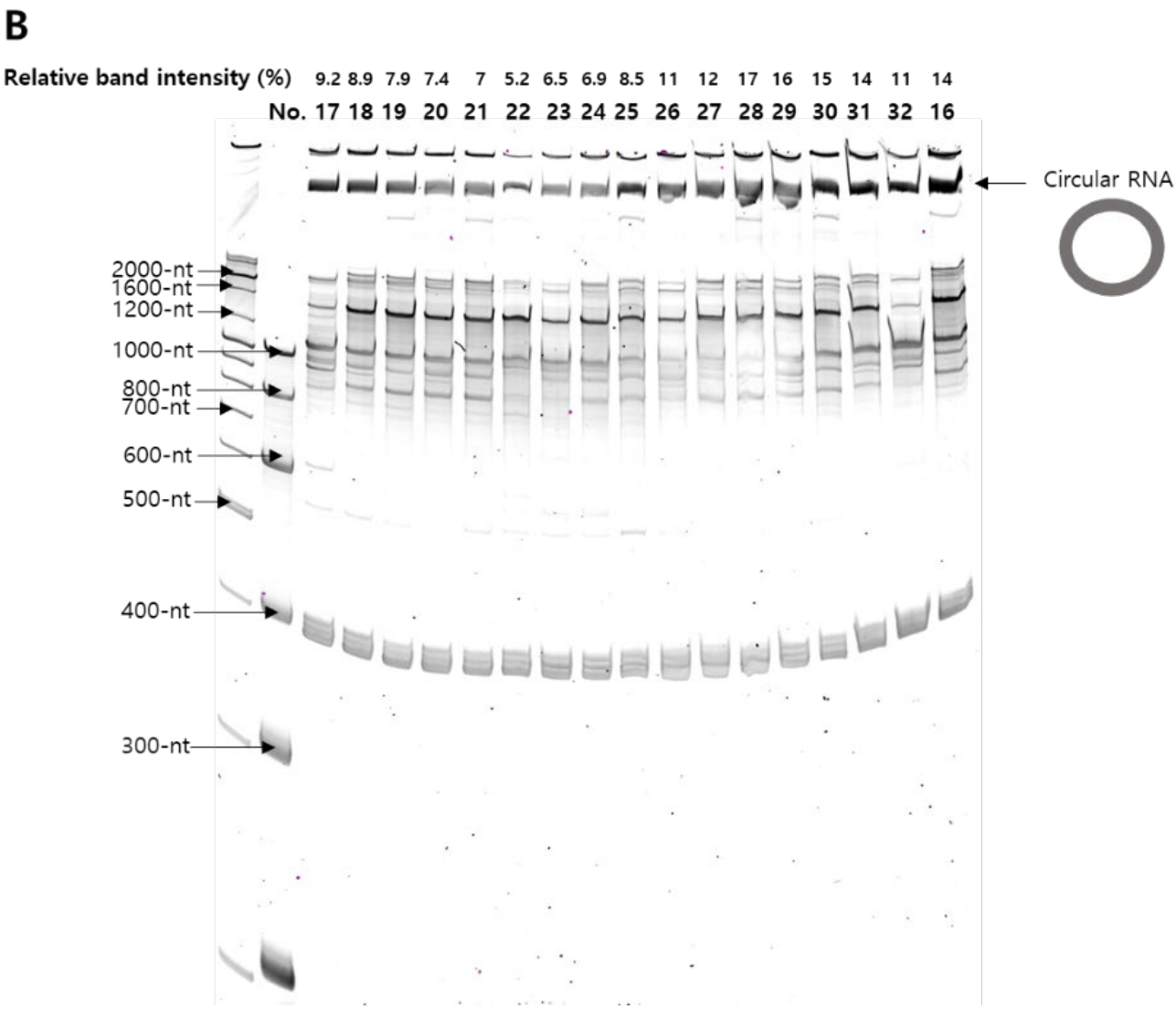

Figure S5. Results of 4% denatured PAGE analysis for IVT samples of self-circularization RNA P1 construct (GOI: EMCV IRES-Gaussia luciferase) with different AU-rich target sequences. (**B**) AU-rich No. 17 to 32 and AU-rich No. 16 RNA constructs. Target sequence of each No. of AU-rich RNA construct is listed in Table S2. Relative band intensity of circRNA is shown as a percentage of whole intensity of each lane.

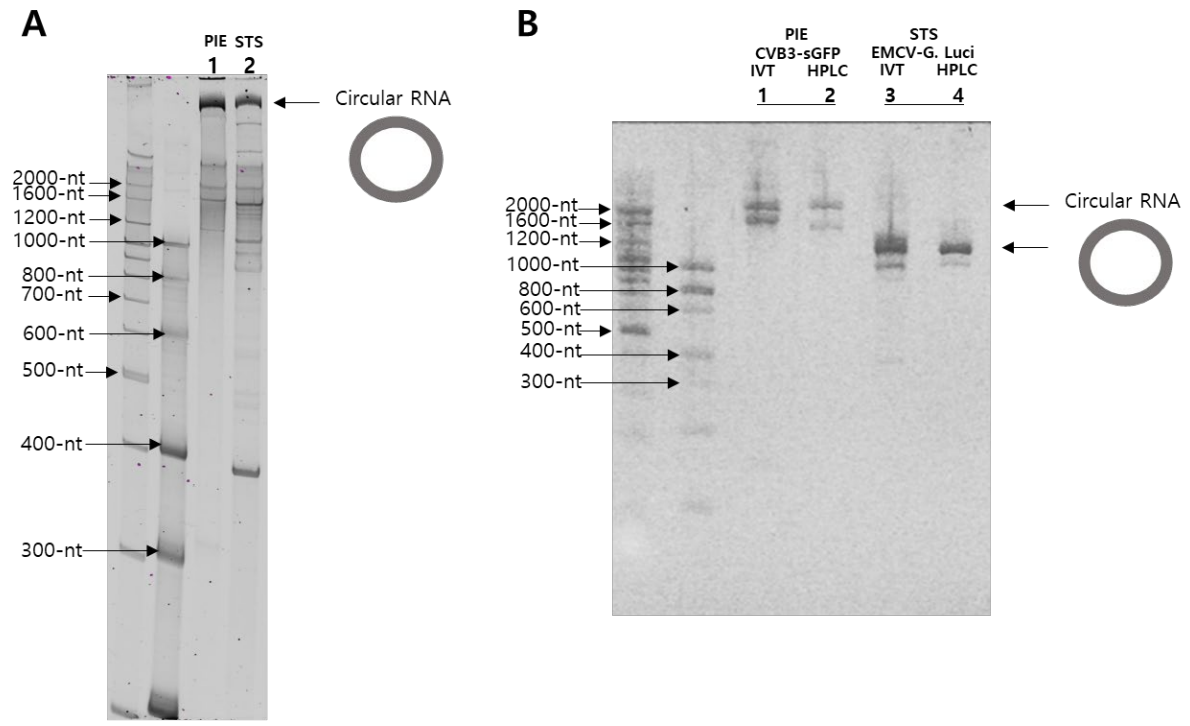

Figure S6. Comparison of circularization efficacy between PIE and end-to-end STS reaction. (**A**) Results of 4% denatured PAGE analysis for IVT samples of PIE construct (lane 1) and end-to-end STS P1 RNA construct (GOI: CVB3 IRES-sGFP) (lane 2). (**B**) Result of 2% E-gel electrophoresis using Ex 1% - 2% program for IVT samples of PIE construct (GOI: CVB3 IRES-sGFP) and end-to-end STS P1 RNA construct (GOI: EMCV IRES-Gaussia luciferase). (lane 1: PIE, lane 2: HPLC-purified PIE circRNA, lane 3: STS, lane 4: HPLC-purified STS circRNA).

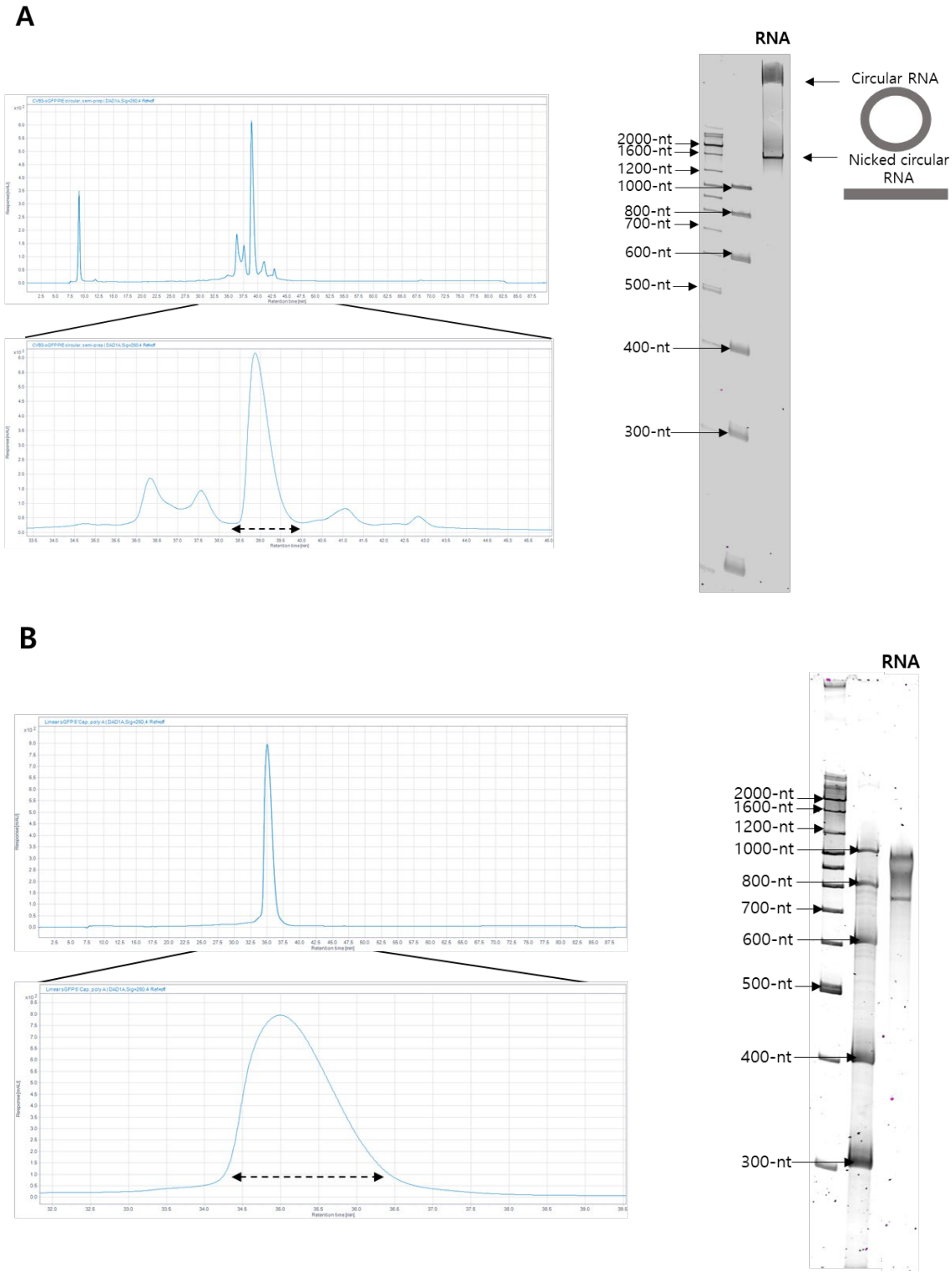

Figure S7. IP-RP HPLC purification of circRNA prepared by PIE or control linear RNA. (**A**) HPLC chromatogram (left) of IVT sample prepared by PIE (GOI: CVB3 IRES-sGFP) and 4% denatured PAGE analysis of the purified circRNA (right). (**B**) HPLC chromatogram of linear m1ψ-modified sGFP with 5′ cap and 3′ polyA (left) and 4% denatured PAGE analysis of the purified linear RNA (right). Eluted region is indicated by a double-headed arrow.

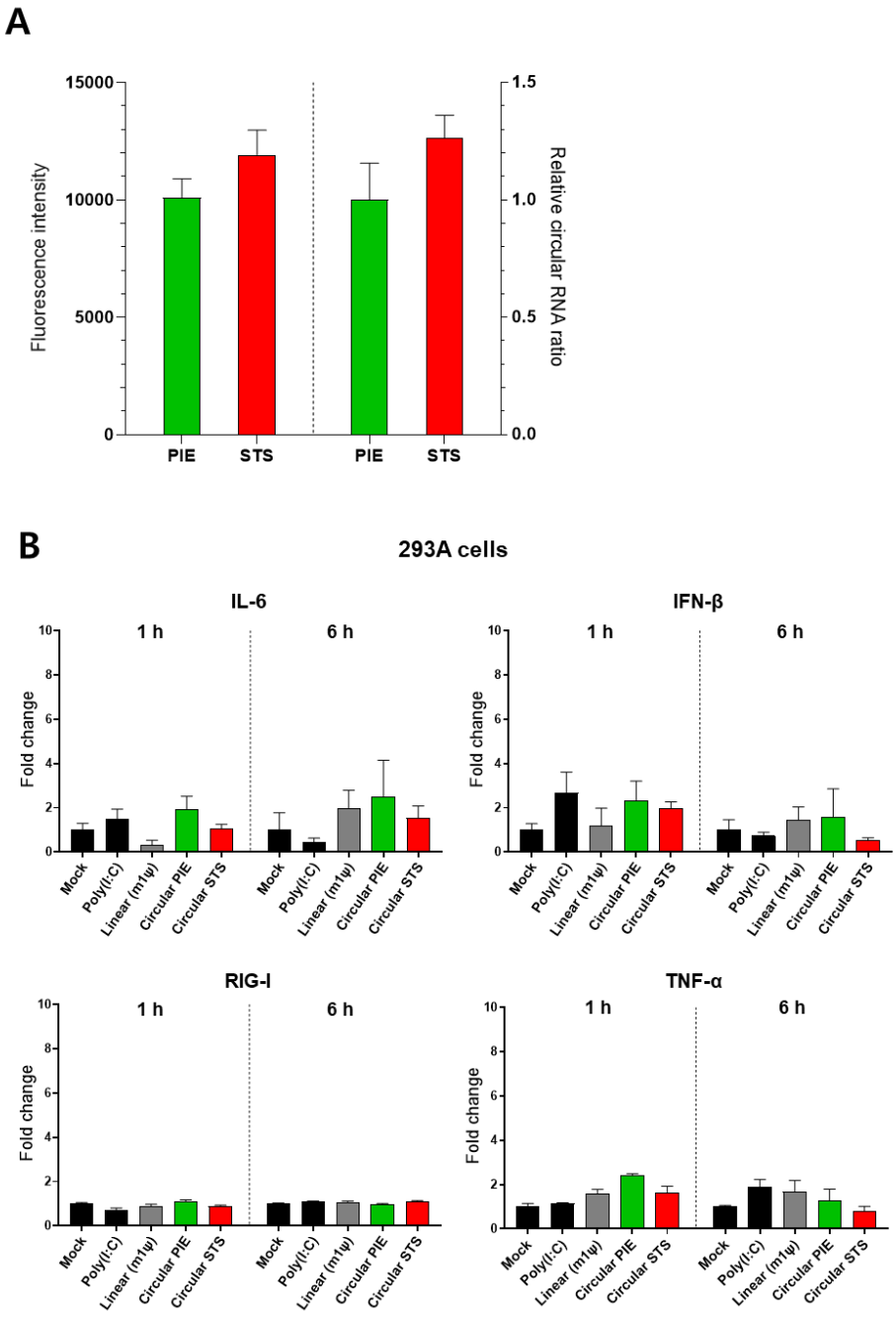

Figure S8. Correlations of protein expression with circRNA quantity and innate immunity induced by circRNA in HEK293A cells. (**A**) Fluorescence intensities of cells (left) and relative quantities of intracellular circRNAs by qRT-PCR using STS primers (right) at 24 h after transfection with equimolar amounts of circRNAs (GOI: CVB3 IRES-sGFP) generated through PIE or end-to-end STS reaction. (**B**) qRT-PCR analysis using specific primers for innate immunity markers with RNAs extracted from transfected HEK293A cells. Poly(I:C) and linear RNA with m1ψ-modified base were used as controls. RNA levels were indicated relative to those in mock-transfected cells. Data are presented as mean ± SEM (n = 3).

Table S1. Sequences of spacers (linkers) between *Tetrahymena* group I intron and IRES.

Spacer (linker)	Sequences (5′ to 3′)
A10	AAAAAAAAAA
A30	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
A50	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
Control 1	GGUAGUGGUGCUACUAACUUCAGCCUGCUGAAGCA
Control 2	GGUAGUAAACUACUAACUACAACCUGCUGAAGCA

Table S2. A total of 32 AU-rich target site sequences (No. 1 – No. 32). No. 17 and 18 target sites exist in ribozyme sequences.

No.	A5 or A4U1	No.	U5 or U4A1
1:	----AAAAAU-3′-end	7:	----UUUUUU-3′-end
2:	----UAAAAU-3′-end	8:	----AUUUUU-3′-end
3:	----AUAAAU-3′-end	9:	----UAUUUU-3′-end
4:	----AAUAAU-3′-end	10:	----UUAUUU-3′-end
5:	----AAAUAU-3′-end	11:	----UUUAUU-3′-end
6:	----AAAAUU-3′-end	12:	----UUUUAU-3′-end

	A2U3		U2A3
13:	----AAUUUU-3′-end	23:	----UUAAAU-3′-end
14:	----AUAUUU-3′-end	24:	----UAUAAU-3′-end
15:	----AUUAUU-3′-end	25:	----UAAUAU-3′-end
16:	----AUUUAU-3′-end	26:	----UAAAUU-3′-end
17:	----UAAUUU-3′-end (In ribozyme)	27:	----AUUAAU-3′-end
18:	----UAUAUU-3′-end (In ribozyme)	28:	----AUAUAU-3′-end
19:	----UAUUAU-3′-end	29:	----AUAAUU-3′-end
20:	----UUAAUU-3′-end	30:	----AAUUAU-3′-end
21:	----UUAUAU-3′-end	31:	----AAUAUU-3′-end
22:	----UUUAAU-3′-end	32:	----AAAUUU-3′-end

Table S3. PCR primers used in experiments.

Primers	Sequences (5′ to 3′)
For DNA construct of circular RNA and RT-PCR of STS product	
T7 F for DNA template	GGGATTCGAACATCGATTAATACGACTCACTATAGGGGCATCGATTGAATTGTCGA
T7 R for DNA template	AGATCTCTCGAGCAGCGCTGCTCGAGGCAAGCTT
T7 F for P1&P10 DNA template	ATAATACGACTCACTATAGGGCGTACTCCGCCCAAAAAAGTTATCA
T7 R for P1&P10 DNA template	CCCACCCAAACCGGTTTTTTTTTTTTTAGTCAC
T7 F for P1 DNA template	ATAATACGACTCACTATAGGGGNNNNNAAAAGTTATCAGGCATGCACCTGGT
T7 R for P1 DNA template	ANNNNNACCGGTTTTTTTTTTTTTAGTCACCACCG
STS F (GOI: EMCV-G. Luci)	CAAGGACTTGGAGCCCATGGAGCAG
STS F2 (GOI: EMCV-G. Luci)	ATGGGAGTCAAAGTTCTGTTTGCCCTGA
STS R (GOI: EMCV-G. Luci)	TGTGCCGCCTTTGCAGGTGTATC
STS F (GOI: CVB3-sGFP)	AGGATGGCAGCGTGCAGCTGGCTGA
STS R (GOI: CVB3-sGFP)	GTCCGGGGTAACAGAAGTGCTTGAT
For qRT-PCR of inflammatory cytokine genes	
18S F	CTTAGAGGGACAAGTGGCG
18S R	ACGCTGAGCCAGTCAGTGTA
TNFα F	TCCCCAGGGACCTCTCTCTA
TNFα R	AGGGTTTGCTACAACATGGGC
IL6 F	AGCCACTCACCTCTTCAGAAC
IL6 R	GCCTCTTTGCTGCTTTCACAC
RIG-I F	TGTGGGCAA TGTCA TCAAAA
RIG-I R	GAAGCACTTGCTACCTCTTGC
IFNβ F	TCTAGCACTGGCTGGAATGAG
IFNβ R	GTTTCGGAGGTAACCTGTAAG