SUPPPLEMENTARY INFORMATION

One-Pot Production of RNA Nanoparticles *via* Automated Processing and Self Assembly

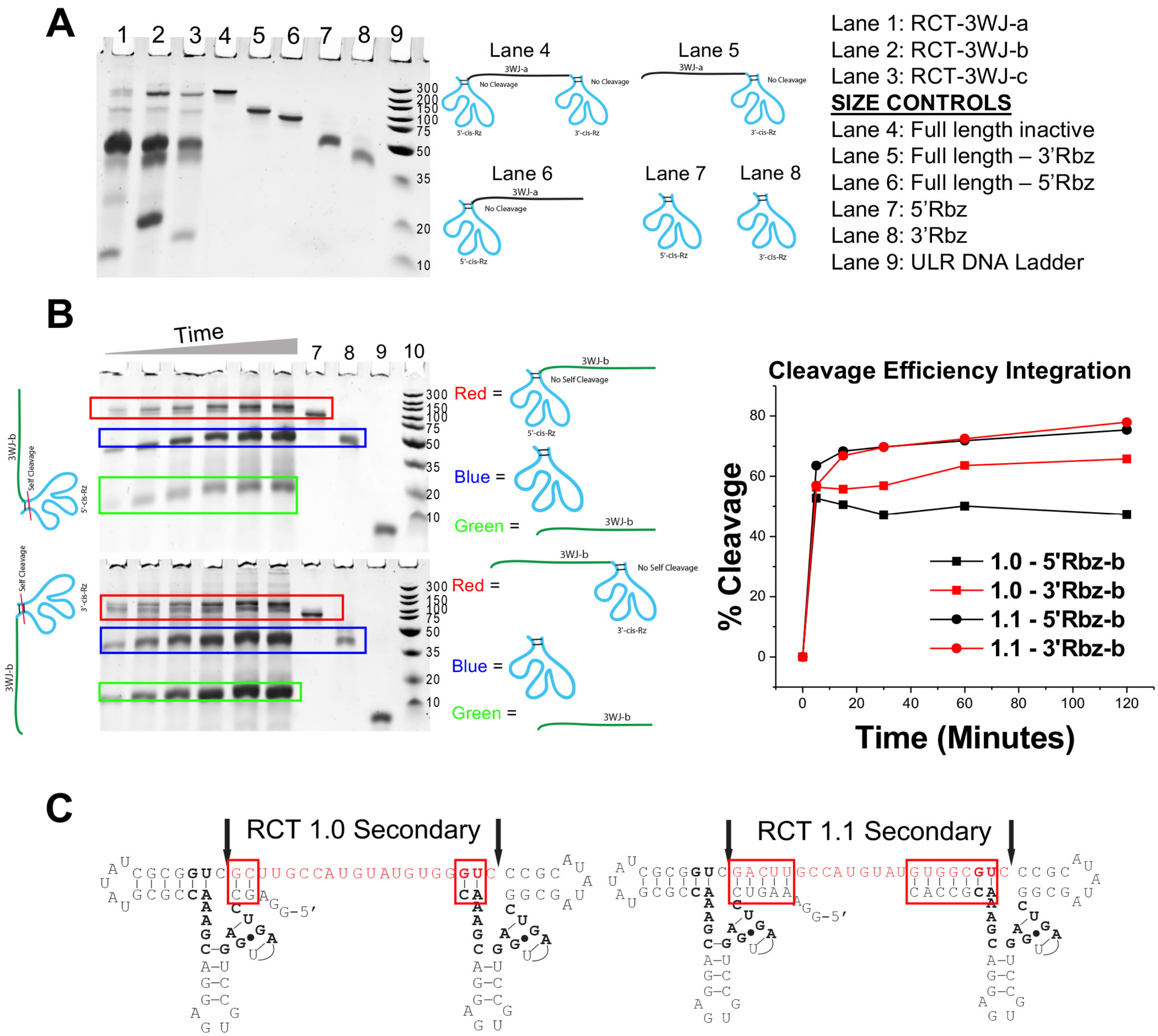
*Daniel L. Jasinski[1, †], Daniel W. Binzel[1, †], and Peixuan Guo[1]**

Center for RNA Nanobiotechnology and Nanomedicine; College of Pharmacy, Division of Pharmaceutics and Pharmaceutical Chemistry; College of Medicine, Department of Physiology & Cell Biology; Dorothy M. Davis Heart and Lung Research Institute; and James Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio 43210, USA.

[†] These authors contributed equally to this work

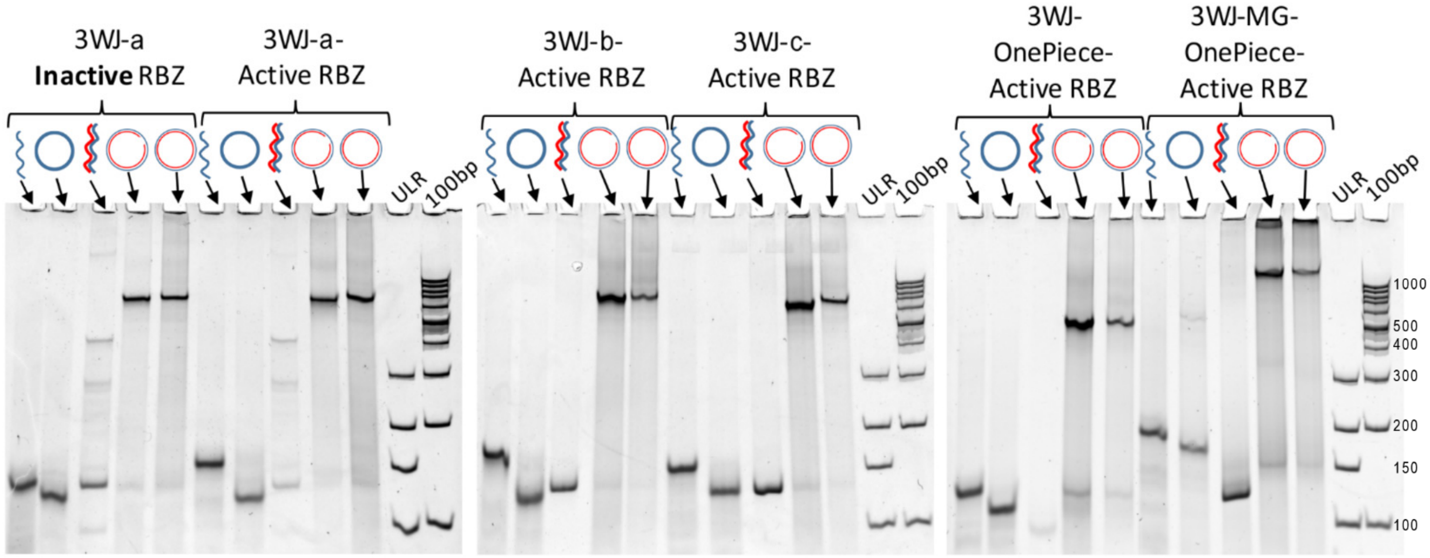
Keywords: rolling circle transcription, RNA nanoparticles, pRNA 3WJ motif, nanotechnology, nanobiotechnology

Supplemental Figure 1: Sequence Design and Ribozyme Optimization



Supp Fig 1. (A) PAGE analysis shows active RCT constructs along with size controls of inactivated ribozyme constructs. Size controls allow confirmation of ribozyme cleavage and release of target RNA oligomers. **(B)** Typical experiment ran to determine cleavage efficiency of self-cleaving ribozymes. The target sequence (green box) intensity was added with the cleaved ribozyme (blue box) intensity and then divided by the total band intensity (red + blue + green box) per well. A plot on the right shows the ribozyme cleavage efficiency over time, comparing first generation design (RCT-1.0) to the second generation design (RCT-1.1). The construct containing 3WJ-b sequence is shown here. While a better curve was desired for ribozyme cleavage kinetics, ribozymes self-cleavage as they are being transcribed, making it difficult to obtain time points of low percentage cleavage. **(C)** An increase in ribozyme efficiency is attributed to increasing the length of the duplex in the "closing" region of the ribozyme sequence, shown in red boxes.

Supplemental Figure 2. Assembly of Circ dsDNA Constructs

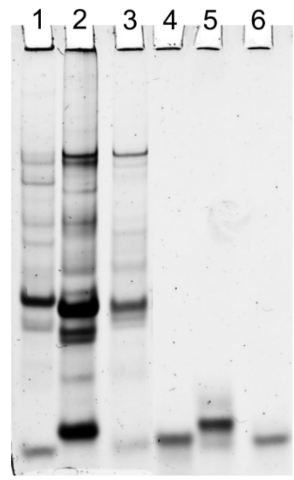


Supp Fig 2. PAGE analyzing the assembly of circular dsDNA constructs containing the T7 promoter used for transcription reactions.

Supplemental Figure 3. Active Ribozyme Constructs, Assembly of Pure Fragments

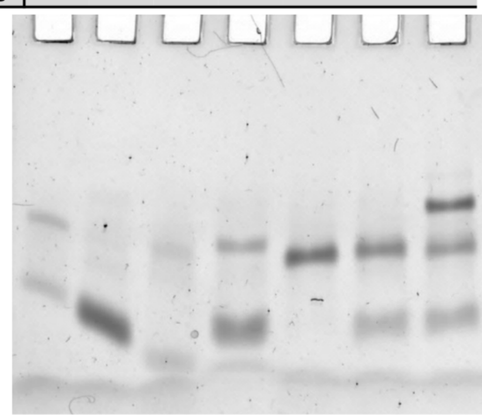
A

Lane 1: Rbz Cleaved 3WJ-a
Lane 2: Rbz Cleaved 3WJ-b
Lane 3: Rbz Cleaved 3WJ-c
Lane 4: 3WJ-a (RCT - synthesized)
Lane 5: 3WJ-b (RCT - synthesized)
Lane 6: 3WJ-c (RCT - synthesized)



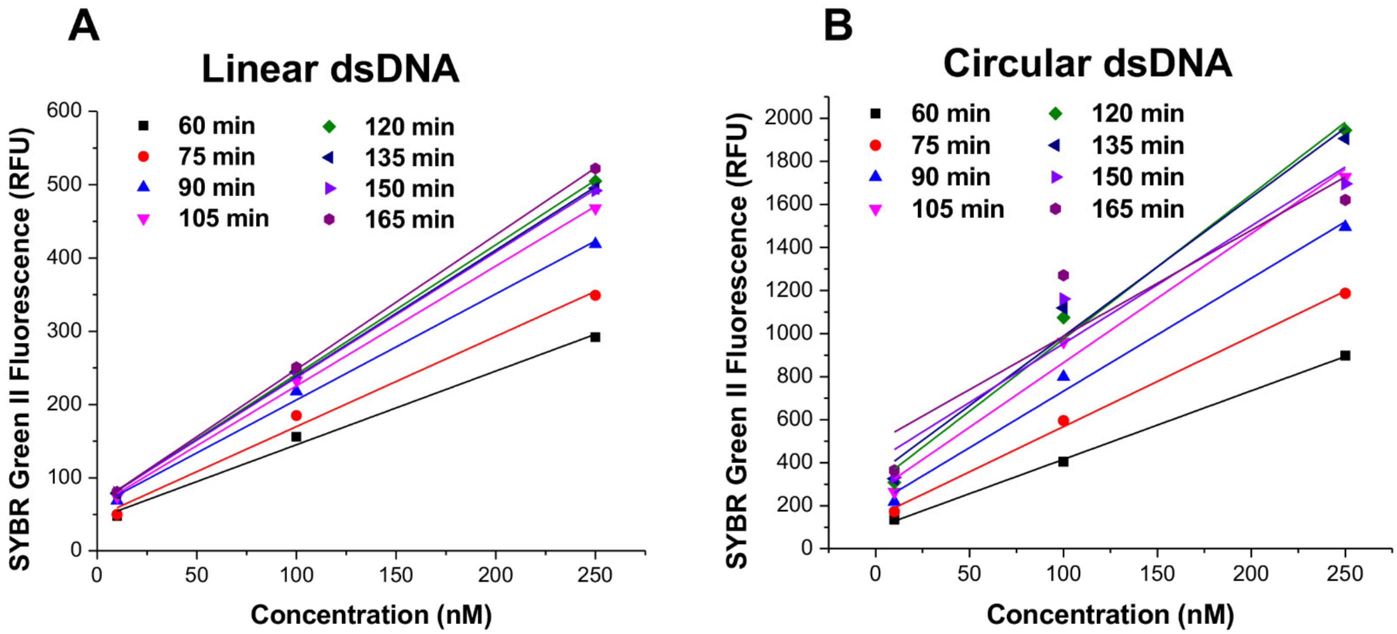
B

	1	2	3	4	5	6	7
RCT-3WJ-a	+			+		+	+
RCT-3WJ-b		+		+	+		+
RCT-3WJ-c			+		+	+	+



Supp Fig 3. (A) Ribozyme cleaved 3WJ ssRNA oligomers were compared to chemically synthesized sequences identical to those of the target sequence. Evidenced by identical migration rate, we can conclude that the cleaved RNA oligomers are the same size as chemically synthesized controls. **(B)** RCT cleaved 3WJ ssRNA oligomers were purified by PAGE band isolation. After elution from gel pieces assembly was tested on native PAGE. A stepwise assembly from monomer to dimer and finally trimer complex demonstrate the ssRNA from RCT reactions are indeed the correct sequence.

Supplemental Figure 4. Fitting Comparison of Concentrations of Transcription Monitoring Experiment



C

	Linear dsDNA					Circular				
	Slope	Standard Error	Intercept	Standard Error	R-Squared	Slope	Standard Error	Intercept	Standard Error	R-Squared
60 min	1.01	0.08	44.68	12.10	0.99	3.19	0.08	97.30	12.28	1.00
75 min	1.23	0.11	47.03	16.77	0.98	4.20	0.20	148.49	31.34	1.00
90 min	1.45	0.08	61.78	13.01	0.99	5.25	0.48	207.95	74.42	0.98
105 min	1.63	0.05	62.25	8.25	1.00	5.99	0.70	265.61	108.79	0.97
120 min	1.77	0.03	64.63	4.12	1.00	6.71	0.72	303.90	111.35	0.98
135 min	1.73	0.04	65.40	6.60	1.00	6.45	0.94	343.37	147.00	0.96
150 min	1.71	0.04	65.95	5.50	1.00	5.47	1.50	406.92	232.78	0.86
165 min	1.83	0.02	64.54	3.39	1.00	4.94	2.05	493.18	318.93	0.71

Supp Fig 4. (A/B) Plots and linear fitting of DNA template concentration, x-axis, *versus* RNA output, as monitored by SYBR GreenII fluorescence. **(C)** Values of slope and intercept, along with their standard errors and R-Squared values of the fits.

Supplemental Table 1. Table of Ribozyme Cleavage Efficiency

	RCT 1.0		RCT 1.1	
	3WJ-a		3WJ-a	
Time (minutes)	5' Rbz	3' Rbz	5'Rbz	3'Rbz
0	0.00%	0.00%	0.00%	0.00%
5	56.7%	25.6%	55.48%	43.54%
15	60.0%	35.5%	61.97%	50.35%
30	61.4%	37.6%	64.04%	50.93%
60	62.9%	49.7%	64.22%	57.94%
120	65.7%	53.3%	68.12%	71.86%
	3WJ-b		3WJ-b	
Time (minutes)	5'Rbz	3'Rbz	5'Rbz	3'Rbz
0	0.00%	0.00%	0.00%	0.00%
5	52.69%	56.35%	63.53%	56.91%
15	50.55%	55.70%	68.33%	66.85%
30	47.18%	56.83%	69.77%	69.66%
60	50.08%	63.56%	71.88%	72.48%
120	47.25%	65.77%	75.37%	77.99%
	3WJ-c		3WJ-C	
Time (minutes)	5'Rbz	3'Rbz	5'Rbz	3'Rbz
0	0.00%	0.00%	0.00%	0.00%
5	16.18%	15.80%	47.43%	45.38%
15	20.89%	16.15%	55.98%	52.39%
30	22.21%	24.59%	56.82%	56.94%
60	25.80%	30.05%	58.45%	59.93%
120	30.20%	36.72%	65.35%	64.66%

Full Length Cleavage	
Construct	**Cleavage %**
RCT-1.1-3WJ-a	87.12%
RCT-1.1-3WJ-b	85.76%
RCT-1.1-3WJ-c	80.83%

Supp Table 1. Table summarizing the cleavage efficiencies of the ribozyme in each of the sequences, broken, down for 5’ and 3’ ribozyme of each sequence, as well as total cleavage efficiency of the full length constructs (those containing both 5’ and 3’ ribozymes).

Supplemental Table 2. Summary of DeltaG of our Circular Constructs and from Other Published Papers

Parameters:	Sequence set to circular, 25 mM NaCl, 6 mM Mg, 37C	mFold Calculation
Publication	**Sequence**	**Delta G (kcal/mol)**
Diegelman, NAR, 1998	ACAACGTGTGTTTCTCTGGTTGACTTCTCTGCTTGCAGGACTGTCAGGAGGTACCAGGTAATA TACC	2.63
Diegelman, NAR, 1998	TTGAAACAGGACTGTCAGGAGGTACCAGGTAATATACCACAACGTGTGTTTCTCTGGTTGACT TCTCTGTTTC	-0.59
Diegelman, NAR, 1998	TGGAACCAGAAACAGGACTGTCATCGAGTACCAGGTAATATACCAAACGTGTGTTTCTCTGGT TGACTTCTCTGTTTC	-3.4
Diegelman, NAR, 1998	CGAAAACTGGACTACAGGGAGGTACCAGGTAATGTACCACAACGTGTGTTTCTCTGGTCTGC TTCTCAGGAAT	4.71
Lindstrom, Biochemistry, 2002	CACTCCACTCACAACATCCACACCTCACACTACAACTCCAACACACTCACTCCT	NO FOLDING POSSIBLE
Lindstrom, Biochemistry, 2002	CCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAA	9.12
Lindstrom, Biochemistry, 2002	CCCACACCCACACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAA	9.12
Hartig, ChemBioChem, 2005	TAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTTAGGGT	8.2
Hartig, ChemBioChem, 2005	TAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTTAGGGT	8.52
Hartig, ChemBioChem, 2005	TAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAG GGTTTAGGGT	8.77
Daubendiek, JACS, 1995	CCTTCTTTCTTTTCCGATCCTTTTCTTTCTTCCT	7.24
Daubendiek, JACS, 1995	CTTTTTTTTTTTTCACACTTTTTTTTTTTTCACA	NO FOLDING POSSIBLE
*Hammond, 2012, Nat. Mat.	ATAGTGAGTCGTATTAACGTA CCAACAACTTACGCTGAGTACTTCGATTACTTGAATCGAAGTACTCAGCGTAAGTTTAGAGGC ATATCCCT	-16.73
*Jang, 2015, Nat. Comm.	ATAGTGAGTCGTATTAACGTACCAACAAGAGAGTTCAAGTCCATCTACAATCTAAAAGTGGTG GGTGTGACCCTAAAATGTAGATGGACTTGAACTCTTTAGAGGCATATCCCT	-11.27
*Jang, 2015, Nat. Comm.	ATAGTGAGTCGTATTAACGTACCAACAAGAGACTTCAAGTGCAACTTCAATCTAAAAGTGGTG GGTGTGACCCTAAAATGAAGTTGCACTTGAAGTCTTTAGAGGCATATCCCT	-12.36
*Roh, 2015, Angew. Chem Int. Ed.	ATAGTGAGTCGTATTAACGTATTAACGTATACCAACCGGCAAGCTGACCCTGAAGTTCACTTG A TGAACTTCAGGGTCAGCTTGCTAGAGGTATTAACGTACATATCCCT	-19.56
*Roh, 2015, Angew. Chem. Int. Ed.	ATAGTGAGTCGTATTAACGCCACAACGTGTACCAACAATGTAGATGGACTTGAACTCACTTGA AGAGTTCAAGTCCATCTACATAGAGGCCACAACGTGCATATCCCT	-11.56
*Furukawa, Bio. Med.Chem. Lett., 2008	GGCCACAGGATCCATTCGTTACCTGGCTCTCGCCAGTCGGGATCCACGTACC	-0.64
This manuscript Sequences		
3WJ-a	ATAGTGAGTCGTATTAGTGGCGTTTCGTCCTCACGGACTCATTAGCCGCTATATGCGGGACG CCACATACACATGGCAAGTCGACCGCGATATACGCGGTTTCGTCCTCACGGACTCATTAGGA CTTTCCT	-11.67
3WJ-a-Inactive-Rbz	ATAGTGAGTCGTATTAGTGGCGTTTCGTCCTCACGGACTCATCAGCCGCTATATGCGGGACG CCACATACACATGGCAAGTCGACCGCGATATACGCGGTTTCGTCCTCACGGACTCATCAGGA CTTTCCT	-11.58
3WJ-b	ATAGTGAGTCGTATTAGATCCGTTTCGTCCTCACGGACTCATCAGCCGCTATATGCGGGACG GATCAACAAAGTATGTGGCGTCGACCGCGATATACGCGGTTTCGTCCTCACGGACTCATCAG GACGCTCCT	-10.81
3WJ-c	ATAGTGAGTCGTATTAGGCAAGTTTCGTCCTCACGGACTCATCAGCCGCTATATGCGGGACT TGCCATGATTGATCCGTCGACCGCGATATACGCGGTTTCGTCCTCACGGACTCATCAGGACG GTCCT	-10.86
3WJ-One Piece	ATAGTGAGTCGTATTACGCGGTTTCGTCCTCACGGACTCATCAGTTGCCATGATTGATCCTCT CGGATCAACAAAGTATGTGGCTCTCGCCACATACACATGGCAAGACCGCGTCCCT	-17.87
3WJ-MG One Piece	ATAGTGAGTCGTATTACGCGGTTTCGTCCTCACGGACTCATCAGTTGCCATGATTGATCCTCT CGGATCAACAAAGTATGTGGCGGATCCATTCGTTACCTGGCTCTCGCCAGTCGGGATCCGCC ACATACACATGGCAAGACCGCGTCCCT	-27.04

Supp Table 2. Summary of sequences used in publications with RCT reactions.[39,62,68,70-71,84-86] The DeltaG of most templates are close to 0 or positive, indicating unstable secondary structure, which is suitable for transcription using bacterial polymerases. Besides dumbbell sequences (denotes by a "*" before the reference), which have previously been shown as suitable substrates for transcription. Templates from this manuscript display a large negative DeltaG value, demonstrating the need for a fully double stranded circular DNA template.

Supplemental Table 3. Table of DNA Sequences Used

Sequences for Linear dsDNA	Promoter, 5'Ribozyme, 3WJ Target RNA, 3'Ribozyme
RCT-1.0-3WJ-a	TAATACGACTCACTATAGGAGCCTGATGAGTCCGTGAGGACGAAACCGCGTATATCGCGGTCGCTTGCCATGTGTATGTGGGGTCGCGCGTATATCGCGCCTGATGAGTCCGTGAGGACGAAAC
RCT-1.0-3WJ-b	TAATACGACTCACTATAGGAGCCTGATGAGTCCGTGAGGACGAAACCGCGTATATCGCGGTCGCCCCACATACTTTGTTGATCCGTCGCGCGTATATCGCGCCTGATGAGTCCGTGAGGACGAAAC
RCT-1.0-3WJ-c	TAATACGACTCACTATAGGAGCCTGATGAGTCCGTGAGGACGAAACCGCGTATATCGCGGTCGCGGATCAATCATGGCAAGTCGCGCGTATATCGCGCCTGATGAGTCCGTGAGGACGAAAC
RCT-1.0-3WJ-a-InactiveRbz	TAATACGACTCACTATAGGAGCCTAATGAGTCCGTGAGGACGAAACCGCGTATATCGCGGTCGCTTGCCATGTGTATGTGGGGTCGCGCGTATATCGCGCCTAATGAGTCCGTGAGGACGAAAC
RCT-1.1-3WJ-a	TAATACGACTCACTATAGGAAAGTCCTGATGAGTCCGTGAGGACGAAACCGCGTATATCGCGGTCGACTTGCCATGTGTATGTGGCGTCCCGCATATAGCGGCTGATGAGTCCGTGAGGACGAAACGCCAC
RCT-1.1-3WJ-b	TAATACGACTCACTATAGGAGCGTCCTGATGAGTCCGTGAGGACGAAACCGCGTATATCGCGGTCGACGCCACATACTTTGTTGATCCGTCCCGCATATAGCGGCTGATGAGTCCGTGAGGACGAAACGGATC
RCT-1.1-3WJ-c	TAATACGACTCACTATAGGACCGTCCTGATGAGTCCGTGAGGACGAAACCGCGTATATCGCGGTCGACGGATCAATCATGGCAAGTCCCGCATATAGCGGCTGATGAGTCCGTGAGGACGAAACTTGCC
RCT-1.1-3WJ-a-InactiveRbz	TAATACGACTCACTATAGGAAAGTCCTAATGAGTCCGTGAGGACGAAACCGCGTATATCGCGGTCGACTTGCCATGTGTATGTGGCGTCCCGCATATAGCGGCTAATGAGTCCGTGAGGACGAAACGCCAC
RCT-Mut-2'F-Activity-Test	TAATACGACTCACTATAGGAGCGTCCTGATGAGTCCGTGAGGACGAAACCGCGTATATCGCGGTAGACGCCACATACTTTGTTGATCCGTACCGCATATAGCGGCTGATGAGTCCGTGAGGACGAAACGGATC
Sequences for Circular dsDNA	Promoter, 5'Ribozyme, 3WJ Target RNA, 3'Ribozyme
RCT_1.1 3WJ-a_IN_Circ_AntiSense	Phos-ATAGTGAGTCGTATTAGTGGCGTTTCGTCCTCACGGACTCATTAGCCGCTATATGCGGGACGCCACATACACATGGCAAGTCGACCGCGATATACGCGGTTTCGTCCTCACGGACTCATTAGGACTTTCCT
RCT_1.1 3WJ-a_IN_Circ_Sense	Phos-AGGAAAGTCCTAATGAGTCCGTGAGGACGAAACCGCGTATATCGCGGTCGACTTGCCATGTGTATGTGGCGTCCCGCATATAGCGGCTAATGAGTCCGTGAGGACGAAACGCCACTAATACGACTCACTAT
RCT_1.1_3WJ-a_Circ_AntiSsnse	Phos-ATAGTGAGTCGTATTAGTGGCGTTTCGTCCTCACGGACTCATCAGCCGCTATATGCGGGACGCCACATACACATGGCAAGTCGACCGCGATATACGCGGTTTCGTCCTCACGGACTCATCAGGACTTTCCT
RCT_1.1_3WJ-a_Circ_Sense	Phos-AGGAAAGTCCTGATGAGTCCGTGAGGACGAAACCGCGTATATCGCGGTCGACTTGCCATGTGTATGTGGCGTCCCGCATATAGCGGCTGATGAGTCCGTGAGGACGAAACGCCACTAATACGACTCACTAT
RCT_1.1_3WJ-b_Circ_AntiSense	Phos-ATAGTGAGTCGTATTAGATCCGTTTCGTCCTCACGGACTCATCAGCCGCTATATGCGGGACGGATCAACAAAGTATGTGGCGTCGACCGCGATATACGCGGTTTCGTCCTCACGGACTCATCAGGACGCTCCT
RCT_1.1_3WJ-b_Circ_Sense	Phos-AGGAGCGTCCTGATGAGTCCGTGAGGACGAAACCGCGTATATCGCGGTCGACGCCACATACTTTGTTGATCCGTCCCGCATATAGCGGCTGATGAGTCCGTGAGGACGAAACGGATCTAATACGACTCACTAT
RCT_1.1_3WJ-c_Circ_AntiSense	Phos-ATAGTGAGTCGTATTAGGCAAGTTTCGTCCTCACGGACTCATCAGCCGCTATATGCGGGACTTGCCATGATTGATCCGTCGACCGCGATATACGCGGTTTCGTCCTCACGGACTCATCAGGACGGTCCT
RCT_1.1_3WJ-c_Circ_Sense	Phos-AGGACCGTCCTGATGAGTCCGTGAGGACGAAACCGCGTATATCGCGGTCGACGGATCAATCATGGCAAGTCCCGCATATAGCGGCTGATGAGTCCGTGAGGACGAAACTTGCCTAATACGACTCACTAT
3WJ_OnePiece_Circ_AntiSense	Phos-ATAGTGAGTCGTATTACGCGGTTTCGTCCTCACGGACTCATCAGTTGCCATGATTGATCCTCTCGGATCAACAAAGTATGTGGCTCTCGCCACATACACATGGCAAGACCGCGTCCCT
3WJ_OnePiece_Circ_Sense	Phos-AGGGACGCGGTCTTGCCATGTGTATGTGGCGAGAGCCACATACTTTGTTGATCCGAGAGGATCAATCATGGCAACTGATGAGTCCGTGAGGACGAAACCGCGTAATACGACTCACTAT
MG-3WJ_OnePiece_AntiSense	Phos-ATAGTGAGTCGTATTACGCGGTTTCGTCCTCACGGACTCATCAGTTGCCATGATTGATCCTCTCGGATCAACAAAGTATGTGGCGGATCCATTCGTTACCTGGCTCTCGCCAGTCGGGATCCGCCACATACACATGGCAAGACCGCGTCCCT
MG-3WJ_OnePiece_Circ_Sense	Phos-AGGGACGCGGTCTTGCCATGTGTATGTGGCGGATCCCGACTGGCGAGAGCCAGGTAACGAATGGATCCGCCACATACTTTGTTGATCCGAGAGGATCAATCATGGCAACTGATGAGTCCGTGAGGACGAAACCGCGTAATACGACTCACTAT
3WJ Sequences	
3WJ-a-RCT	GACUUGCCAUGUGUAUGUGGCGUC
3WJ-b-RCT	GACGCCACAUACUUUGUUGAUCCGUC
3WJ-c-RCT	GACGGAUCAAUCAUGGCAAGUC