

Supporting Information

**Selective Nonenzymatic Formation of Biologically Common RNA Hairpins**

*L.-F. Wu, J. Zhang, R. Cornwell-Arquitt, D. A. Hendrix, A. Radakovic, J. W. Szostak\**

**Supporting information for**  
**Selective Nonenzymatic Formation of Biologically Common**  
**RNA Hairpins**

Long-Fei Wu<sup>1, †</sup>, Junting Zhang<sup>2</sup>, Robert Cornwell-Arquitt<sup>3</sup>, David Hendrix<sup>3,4</sup>,  
Aleksandar Radakovic<sup>1</sup> and Jack W. Szostak<sup>1,\*</sup>

<sup>1</sup> Howard Hughes Medical Institute, The University of Chicago, Chicago, IL 60637, USA.

<sup>2</sup> The University of Chicago, Chicago, IL 60637, USA.

<sup>3</sup> Department of Biochemistry and Biophysics, Oregon State University, USA

<sup>4</sup> School of Electrical Engineering and Computer Science, Oregon State University, USA

<sup>†</sup>Current address: Frontiers Science Center for Transformative Molecules, School of Chemistry and Chemical Engineering, Shanghai Jiao Tong University, Shanghai, 200240, China

\*Correspondence to: [jwszostak@uchicago.edu](mailto:jwszostak@uchicago.edu)

**The PDF file includes:**

Materials and General.....	2
Supplementary Methods.....	3
Supplementary Figure S1 to S14 .....	10
Supplementary Table S1 to S8 .....	24
Appendix S1 to S2 .....	73
Uncropped, full-size gel images.....	74

## Materials and General

For solid-phase RNA synthesis, 1  $\mu$ mol scale pre-packed synthesis columns were acquired from Glen Research. Phosphoramidites utilized for RNA synthesis were sourced from Glen Research and ChemGenes. RNA oligomers were synthesized using either a K&A H-8 DNA/RNA Synthesizer or an Expedite 8909 at 1  $\mu$ mol scale, or alternatively purchased from Integrated DNA Technologies (IDT). To synthesize randomized nucleobase in an oligo, an input ration of premixed phosphoramidites of A:C:G:U = 3:3:2:2 was used. The MiSeq Reagent Kit v3 (150-cycle) was obtained from Illumina. T4 RNA Ligase 2 truncated K227Q, ProtoScript II Reverse Transcriptase, and Q5 Hot Start High-Fidelity DNA Polymerase were procured from New England Biolabs (NEB). Amicon® Ultra-4 Centrifugal Filters, 10 kDa MWCO, were acquired from MilliporeSigma. High-pressure liquid chromatography (HPLC) analysis was performed using a Nexera HPLC/UHPLC system (Shimadzu) equipped with an Atlantis™ T3, 3  $\mu$ m, 4.6 x 150 mm column, and detection was achieved at 260 nm using an SPD-M30A UHPLC Photodiode Array Detector. Polyacrylamide gel electrophoresis was conducted on 8% to 20% polyacrylamide, 8 M urea gels (0.75 mm thick, 20 cm long) at 18 W in 1X TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3) for 1 to 2 hours. FAM-labelled RNA oligomers were detected and imaged with an Amersham RGB Biomolecular Imager (GE Healthcare Life Science, Marlborough, MA) and quantified using the ImageQuant™ software package (GE Healthcare Life Science, Marlborough, MA). For imaging unlabelled RNA oligos, the gel was stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen). Oligonucleotide concentrations were determined by UV absorbance at 260 nm using a NanoDrop® ND-1000 spectrophotometer.

## Supplementary Methods

### General procedure for chemical synthesis of 5'-phosphorimidazolidine RNA (Im-p-RNA) from 5'-phosphate RNA (5'-p-RNA).

A 300  $\mu$ L aqueous reaction mixture containing 0.1 mM 5'-p-RNA and 100 mM imidazole (pH 7.0) was prepared. To this mixture, 5.8 mg of EDC was added to achieve a final concentration of 80 mM, and the mixture was incubated at room temperature. After 2 hours, 10 mL of cold 50 mM NaClO<sub>4</sub> in acetone was added, creating a cloudy mixture that was vigorously vortexed and then chilled on dry ice for 30 minutes. The mixture was then centrifuged at 4000 rpm for 10 minutes at 4°C, yielding a white pellet. The supernatant was discarded, and the pellet was resuspended in 2 mL of cold 50 mM NaClO<sub>4</sub> in acetone, vortexed extensively for 30 seconds, and centrifuged again under the same conditions. This washing step was repeated twice. The pellet was then dried in a SpeedVac under vacuum without heat for 30 minutes. The resulting white pellet was redissolved in 60  $\mu$ L of 20 mM HEPES buffer (pH 8.0) and stored at -80°C for future use without further purification. The concentration of Im-p-RNA combined with 5'-p-RNA was quantified by UV absorbance at 260 nm using a NanoDrop. The conversion from 5'-p-RNA to Im-p-RNA was assessed by HPLC analysis, employing a UV detection system at 260 nm. The system used an Atlantis<sup>TM</sup> T3, 3  $\mu$ m, 4.6 x 150 mm column, with an 8  $\mu$ L injection of 10  $\mu$ M RNA solution. The flow rate was set at 0.5 mL/min, utilizing LC solvents comprising 50 mM triethylammonium acetate in water (pH 7.5, solvent A) and acetonitrile (solvent B). The mobile phase gradient was programmed as follows: 6% B from 0 to 2 minutes, adjusting to 20% B (dependent on oligo length) by 20 minutes, escalating to 95% B from 22 to 25 minutes, and reverting to 6% B from 27 to 30 minutes before ending the analysis. The column compartment temperature was maintained at 25°C.

### **Loop-closing ligation using randomized NNNN overhangs.**

10  $\mu$ L reaction mixture, containing phosphate acceptor strand A (5 nM or 250 nM, X = A, C, G, or U, respectively, in strand A, see Table S1), pre-activated phosphate donor strand a (6 nM or 300 nM, Y = A, C, G, or U in strand a, see Table S1), Blocker-1 (6 nM or 300 nM), Blocker-2 (6 nM or 300 nM), MgCl<sub>2</sub> 50 mM, HEPES 100 mM (pH 8.0) and *N*-methylimidazole 50 mM (MeIm, pH 8.0, added at last), was incubated for 20 hours at 23 °C. 1  $\mu$ L of each reaction mixture was quenched by adding it to 9  $\mu$ L of stop solution, which consisted of 50 mM EDTA, pH 8.0, in 90 % formamide. Subsequently, 2  $\mu$ L of the quenched solution was analysed using a 10 % analytic denatured PAGE gel. Yields were quantified based on the relative amounts of FAM-labelled oligomers, as determined by gel imaging.

For sequencing purposes, reactions initiated at a concentration of 5 nM were scaled up to 10 mL under similar conditions (see below for follow-up processes).

### **Determining the initial distribution of NNNN overhang sequences.**

The initial distribution of overhang sequences in the as synthesized acceptor strand was determined by T4 RNA ligase catalyzed ligation followed by sequencing (Figure S3). A 22  $\mu$ L reaction mixture was prepared containing 2  $\mu$ M phosphate acceptor strand A (X = A, C, G, or U in strand A, as detailed in Table S1) and 5  $\mu$ M pre-adenylated phosphate donor strand a', along with 1X T4 RNA ligase reaction buffer (NEB: B0216SVIAL) and 20% PEG 8000 (NEB: B1004SVIAL). The mixture was heated to 90°C for 3 minutes and then cooled to 25°C. Subsequently, 3  $\mu$ L of T4 RNA Ligase 2 truncated K227Q at 200 U/ $\mu$ L (NEB: M0351L) was added, resulting in a final enzyme concentration of 24 U/ $\mu$ L. The reaction was incubated at 25°C for 18 hours. To quench the reaction, 250  $\mu$ L of stop solution (50 mM EDTA, pH 8.0, in 90% formamide) was added. The quenched solution was then loaded onto a 10% preparative PAGE gel.

### **Purification of ligation product of large-scale reactions**

10 mL solution from the large-scale loop-closing ligation was concentrated to approximately 250  $\mu$ L using an Amicon® Ultra-4 Centrifugal Filter with a 10 kDa MWCO (MilliporeSigma: UFC801024). The concentrated solution was transferred to a 1.5 mL Eppendorf tube, to which 900  $\mu$ L of cold absolute ethanol and 120  $\mu$ L of 3 M sodium acetate, pH 5.5, were added. The mixture was then placed in a -80 °C freezer or on dry ice for 30 minutes, followed by centrifugation at 15,000 rpm for 15 minutes at 4°C, after which the supernatant was discarded. The pellet was washed twice with 100  $\mu$ L of cold 75% ethanol, centrifuged each time at 15,000 rpm for 10 minutes at 4°C, and then dried for 20 minutes under vacuum using a SpeedVac at room temperature. The dried pellet was resuspended in 50  $\mu$ L of stop solution (50 mM EDTA pH 8.0 in 90% formamide) and prepared for loading onto a preparative RNA PAGE gel. A 10% denatured PAGE gel with a 1.5 mm thickness was cast, featuring 6 wells (well dimensions: 25 mm width by 15 mm depth). The wells were thoroughly cleaned prior to pre-running and loading the samples. RNA samples were loaded, and the gel was run at 20 W for 2 hours after pre-running the gel for 25 minutes at 15 W in 1X TBE buffer. The target band was visualized using a blue light illuminator (Invitrogen) and excised into a 15 mL tube with a clean razor blade. The excised gel slice was physically crushed and mixed with 10 mL of 5 mM EDTA buffer (pH 8.0). The mixture was spinned at 25 rpm overnight at room temperature. Subsequently, the supernatant was filtered through a 0.5  $\mu$ m filter into an Amicon® Ultra-4 Centrifugal Filter, 10 kDa MWCO, using a syringe. The solution was concentrated to 250  $\mu$ L and subjected to ethanol precipitation as previously described. The final dried pellet was redissolved in 30  $\mu$ L of ddH<sub>2</sub>O. The RNA concentration was measured using a NanoDrop and found to be approximately 1-10 ng/ $\mu$ L.

### **Deconvoluting loop-closing ligation and nicked duplex ligation**

We used four strand loop-closing ligation reactions to deconvolute loop closing ligation from nicked duplex ligation (Figure 2 and Figure S6). 10  $\mu$ L reaction mixture, containing phosphate acceptor strand A (5 nM or 250 nM, X = A, C, G, or U in strand A, see Table S1), pre-activated phosphate donor strand a (6 nM or 300 nM, X = A, C, G, or U in strand a, see Table S1), phosphate acceptor strand B (5 nM or 250 nM), pre-activated phosphate donor strand b (6 nM or 300 nM), Blocker-1 (12 nM or 600 nM), Blocker-2 (12 nM or 600 nM), MgCl<sub>2</sub> 50 mM, HEPES 100 mM (pH 8.0) and *N*-methylimidazole (50 mM, pH 8.0, added at last) was incubated for 20 hours at 23 °C. 1  $\mu$ L of each reaction mixture was quenched by addition to 9  $\mu$ L of stop solution (50 mM EDTA pH 8.0 in 90 % formamide). 2  $\mu$ L of the quenched solution was analysed by 10 % analytic PAGE gel. Observed yields were quantified according to the relative amounts of FAM-labelled oligomers by gel imaging.

For sequencing, the reactions at 5 nM were scaled up to 10 mL with similar condition.

### **Preparing the multiplexed DNA library for deep sequencing**

For sequencing library preparation see Figure S1, steps 1 to 5.

**Step 1.** The pre-activated 5'-phosphorimidazolide RNA, Im-p-RNA, was synthesized as described as above.

**Step 2.** Large-scale loop-closing ligation reactions were conducted, and the ligated products were purified by RNA preparative PAGE gel, as detailed above.

**Step 3.** A 50  $\mu$ L aqueous solution containing approximately 100 ng of gel-purified RNA (ligation product), 5  $\mu$ M RT primer (Table S1), 1x ProtoScript II Buffer, 5 mM dNTPs, 10 mM MgCl<sub>2</sub>, 10 mM DTT, and ProtoScript II Reverse Transcriptase (final enzyme concentration of 8 U/ $\mu$ L) (NEB: M0368L) was incubated at 42°C for 12 hours. Then, the solution was heated to 80°C for 5 minutes and then maintained at 4°C. Subsequently, 8  $\mu$ L of 1M NaOH was added, and the mixture was heated at 90°C for 5 minutes, followed by the addition of 8  $\mu$ L of 1M HCl. The resulting solution was processed

through a Zymo Oligo Clean & Concentrate spin column according to the manufacturer's instructions. The cDNA was eluted in 20  $\mu$ L of TE buffer (pH 7) and stored at 4°C. The cDNA stock concentration, typically in the low ng/ $\mu$ L range, was measured by NanoDrop.

**Step 4.** A 25  $\mu$ L solution, consisting of 100 ng cDNA, 0.2 mM dNTP, 0.5  $\mu$ M each of Primer\_P7 (NEBNext Index primer for Illumina, Table S1) and Primer\_P5 (NEBNext SR primer for Illumina, Table S1), 1x Q5 reaction buffer, and Q5 Hot Start High-Fidelity DNA Polymerase (0.8 U total) was subjected to 6 PCR cycles with a 15-second extension step at 62°C. After amplification, 5  $\mu$ L of Gel Loading Dye, Purple (6X) (NEB: B7024S) was added, and the entire volume was run on a preparative 1.4% w/v agarose gel in 1X TAE. Gel solution was prepared as 1.4% w/v agarose in 1X TAE (Tris Acetate-EDTA) and SYBR Safe DNA Gel Stain (5  $\mu$ L in 50 mL gel solution, Invitrogen: S33102). The gel, cast as 10  $\times$  5 cm with large wells capable of holding 50  $\mu$ L of sample, was run at 100 V for 90 minutes at constant voltage. Target bands were visualized using a blue light transilluminator, excised, and purified using a Quantum Prep Freeze'N Squeeze spin column (Bio-Rad). The eluate was further purified using Agencourt AMPure XP magnetic beads with a 1.7:1 bead to sample volume ratio. The purified target material was eluted in 20  $\mu$ L TE (pH 7) and prepared for sequencing.

**Step 5.** Samples were validated by TapeStation (Agilent) and qPCR before sequencing by Illumina MiSeq.

### **Sequencing data analysis and verification of sequencing results**

**Step 6 (Figure S1).** The raw sequence data were processed using a custom Python program, tailored specifically for this study. The final high-quality sequencing reads were derived through stringent trimming criteria, ensuring robustness in downstream analyses. The criteria employed were as follows: 1) the quality score of both R1 and R2 reads must be at least 30; 2) fixed regions within R1 and R2 must exactly match the

expected sequences; 3) R1 and R2 sequences should be reverse complements of each other. The outcomes of the trimming process are detailed in Tables S2, S3, and S4.

**Step 7 (Figure S1).** Test oligonucleotides are designated as Test-NNNN in Table S1. A 10  $\mu$ L reaction mixture, consisting of 1  $\mu$ M preactivated phosphate donor strand Test-donor (Im-p-GCACGAGU) and one of the phosphate acceptor strands, Test-NNNN (1.5  $\mu$ M, sequences 5'-FAM-UUACUCGUGCNNNN-3' or 5'-FAM-AUCGUGCNNNN-3' as listed in Table S1), along with 50 mM MgCl<sub>2</sub>, 100 mM HEPES (pH 8.0), and 50 mM *N*-methylimidazole (added last), was incubated at 23°C for 10 hours. Each reaction mixture was subsequently quenched by adding 1  $\mu$ L to 9  $\mu$ L of stop solution (50 mM EDTA, pH 8.0, in 90% formamide). 2  $\mu$ L of each quenched sample was analyzed on a 10% analytical denatured PAGE gel. Observed yields were quantified based on the relative amounts of FAM-labelled oligomers, as detected by gel imaging. The yields of the 34 tested overhang sequences were used to generate a ranking order, which was then compared with their ranking from the NGS data. Spearman's ranking correlation coefficient was calculated to be  $\rho = 0.84$  (details see Appendix S1). Results are presented in Figure 4.

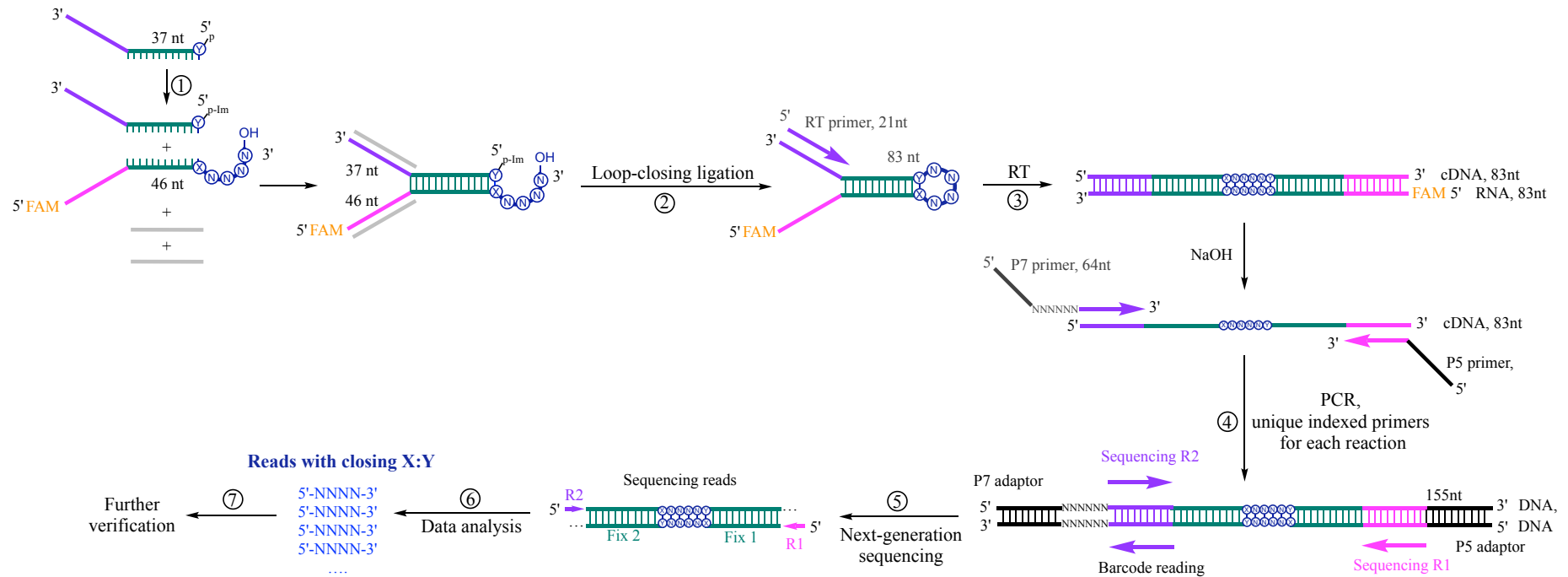
#### **Assembly of the full-length Hammerhead ribozyme via single loop-closing ligation**

A high-yielding sequence, UUCG, was engineered into the Hammerhead ribozyme to optimize the loop-closing ligation efficiency. The reaction was carried out in a 10  $\mu$ L mixture containing 1  $\mu$ M of 5'-FAM-CCUGUCUGAUGAGUCCUUCG-3' (HH-5'-half) and 2  $\mu$ M of 5'-Im-p-GGACGAAACCGU-3' (Im-p-HH-3'-half, including both Im-p-RNA and p-RNA), MgCl<sub>2</sub> (either 5 mM or 50 mM), 100 mM HEPES buffer (pH 8.0), and 50 mM *N*-methylimidazole (added last). The reaction was incubated at 23°C. At specified time points, 1  $\mu$ L aliquots were taken and quenched in 9  $\mu$ L of stop solution (50 mM EDTA, pH 8.0, in 90% formamide). 2  $\mu$ L of each quenched sample was analyzed on a 20% denatured PAGE gel. Observed yields were quantified by comparing the relative amounts of FAM-labelled oligomers through gel imaging.

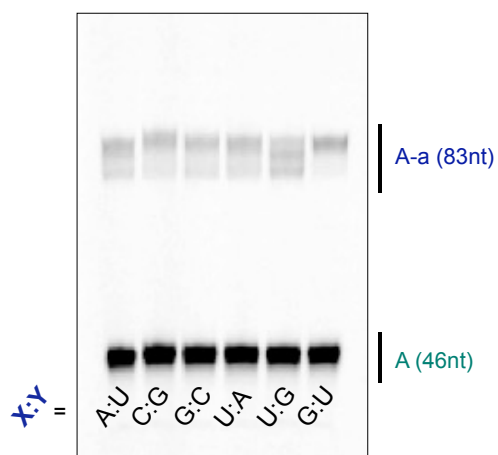
### **Assembly of the full-length Flexizyme via two simultaneous loop-closing ligations**

A high-yielding sequence, UUCG, was integrated into the two hairpin loop regions of the Flexizyme to optimize ligation efficiency. The reaction was carried out in a 10  $\mu$ L mixture containing 1  $\mu$ M 5'-FAM-GGACCUUCG-3' (Flex1), 2  $\mu$ M 5'-Im-p-GGUCCCGCAUCCCAUCUUCG-3' (Im-p-Flex2, including Im-p-RNA and p-RNA), and 5  $\mu$ M 5'-Im-p-GAUGGUACAUGGCGUUAGGU-3' (Im-p-Flex3, including Im-p-RNA and p-RNA), 50 mM MgCl<sub>2</sub>, 100 mM HEPES buffer (pH 8.0), and 50 mM *N*-methylimidazole (added last). The reaction was incubated at 23°C. At specified time points, 1  $\mu$ L aliquots were taken and quenched in 9  $\mu$ L of stop solution (50 mM EDTA, pH 8.0, in 90% formamide). 2  $\mu$ L of each quenched sample was analyzed on a 20% denatured PAGE gel. Observed yields were quantified by comparing the relative amounts of FAM-labelled oligomers through gel imaging.

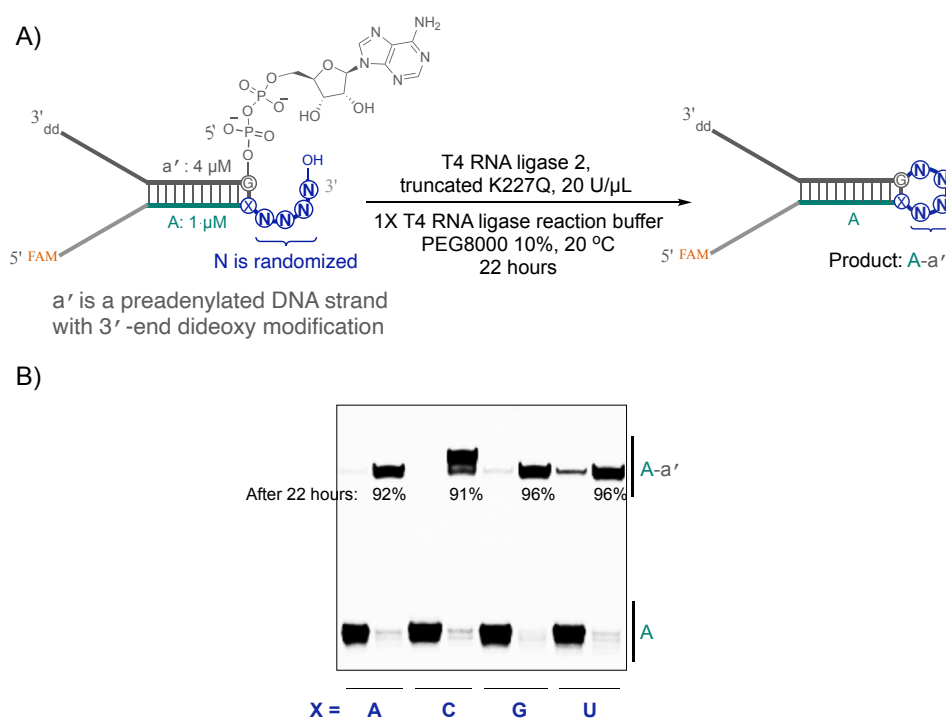
## Supplementary Figures



**Figure S1. Outline of the sequencing workflow used in this work.** Step 1, Chemical synthesis of Imp-RNA from p-RNA. Step 2, Ligated products from large-scale loop-closing ligation purified by prep-PAGE gel. Step 3, cDNA preparation through reverse transcription followed by NaOH treatment. Step 4, Indexed PCR performed using indexed primers. Step 5, Pooling of the indexed DNA library for next-generation-sequencing. Step 6, Data analysis using custom Python program. Step 7, Verification of sequencing results by additional testing.



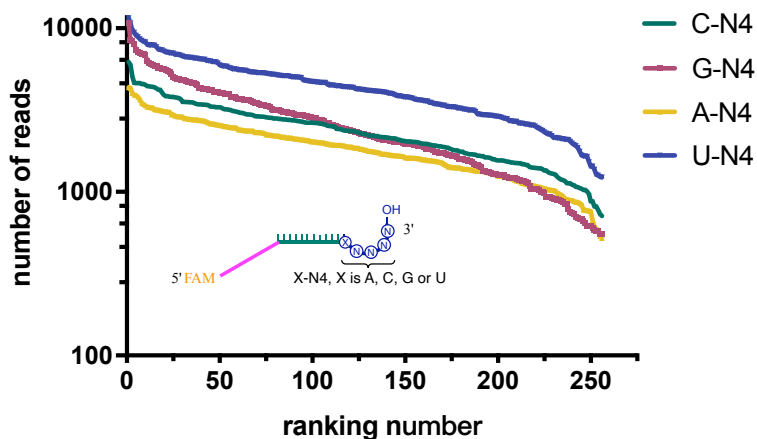
**Figure S2. Representative denatured RNA PAGE gel.** This figure displays the results for reactions with 250 nM duplex A:a, as shown in Figure 2. Uncropped image can be found in the end of this document.



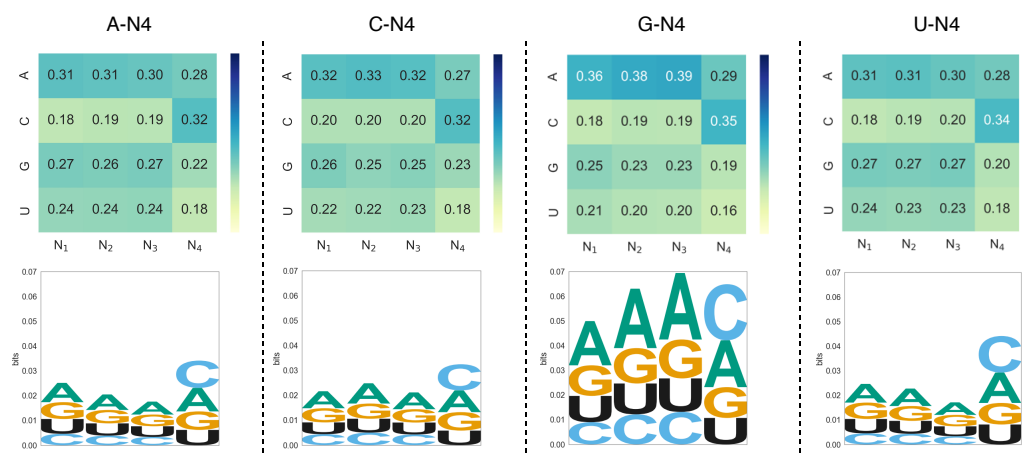
**Figure S3. Loop-closing ligation by T4 RNA ligase.** A) Reaction scheme and condition. The 3'-end nucleoside of strand a' features a dideoxy modification to prevent undesired ligation reaction. Possible splint ligation product is omitted in the scheme. B) Representative denatured RNA PAGE gel and ligation yields. Yields at 1 min and 22

hours are shown. The ligated products were purified and subjected to the sequencing workflow, from step 3 to step 7 as outlined in Figure S1. Uncropped image can be found in the end of this document.

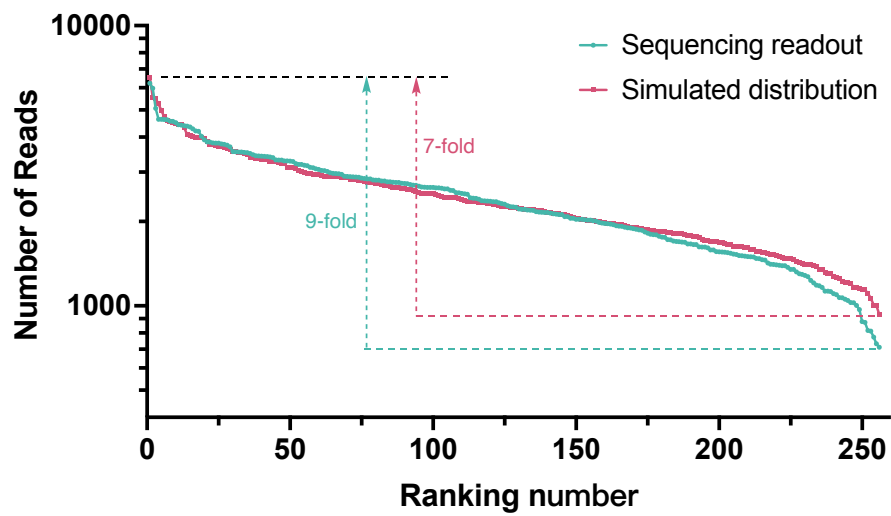
A)



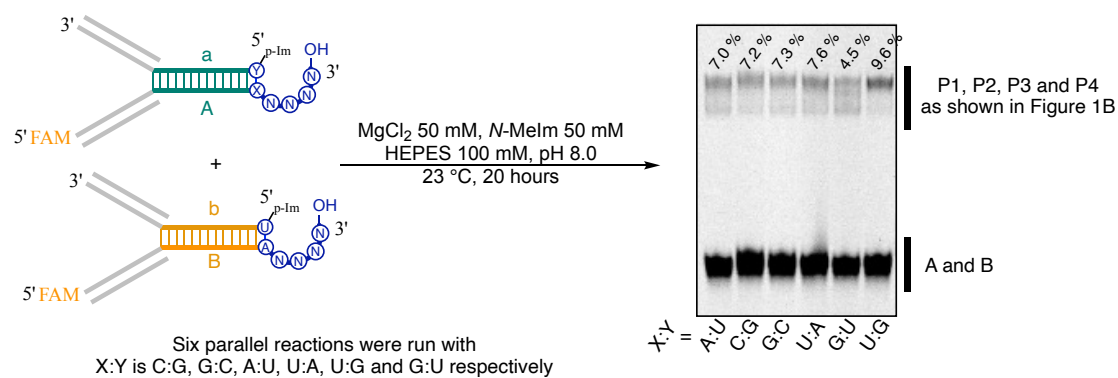
B)



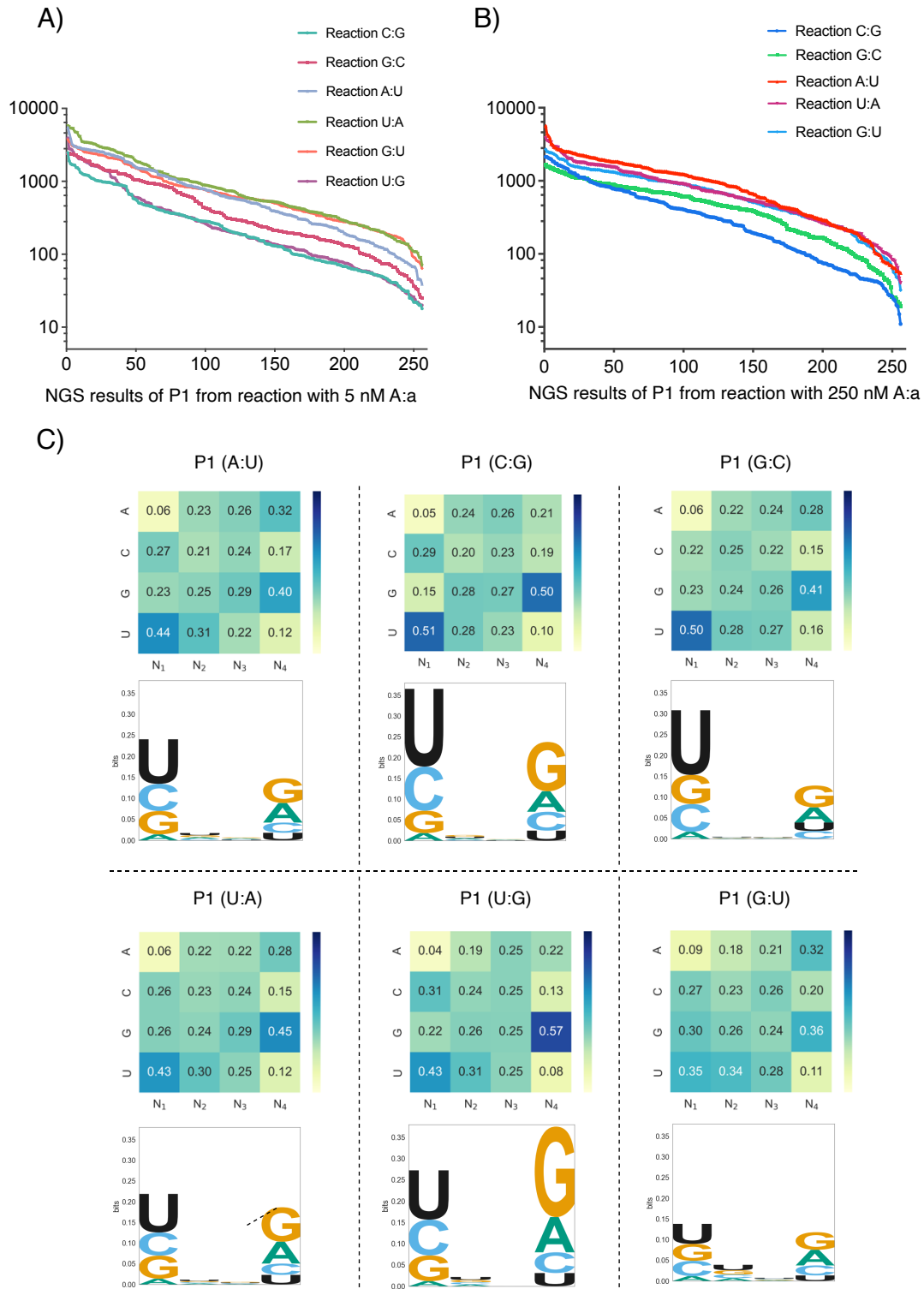
**Figure S4. Distribution of randomized NNNN overhang sequences in the synthesized oligonucleotide.** A) Number of sequencing reads relative to their ranking number, derived from the products of reactions shown in Figure S3. B) Heat maps and sequence consensus logos of the four NNNN overhangs display the distribution of the four nucleotides at each position within the starting materials (information content from 0 to 2 bits). This visual representation highlights the imbalance among nucleotides at each position.



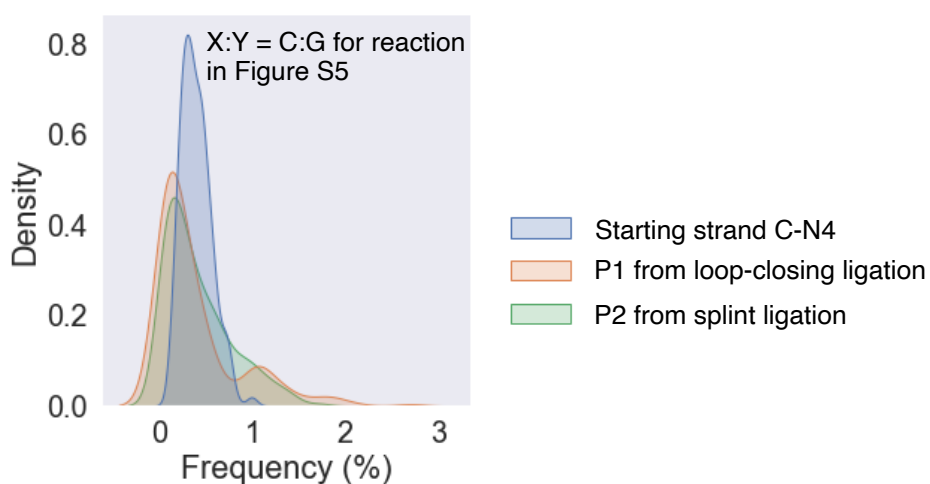
**Figure S5. The variation in abundance of the randomized NNNN results from the biased base-composition at each position.** The base compositions obtained for each position, as depicted in the heatmap of C-N4 in Figure S4, effectively simulate the uneven distribution observed in the NGS results for C-N4.



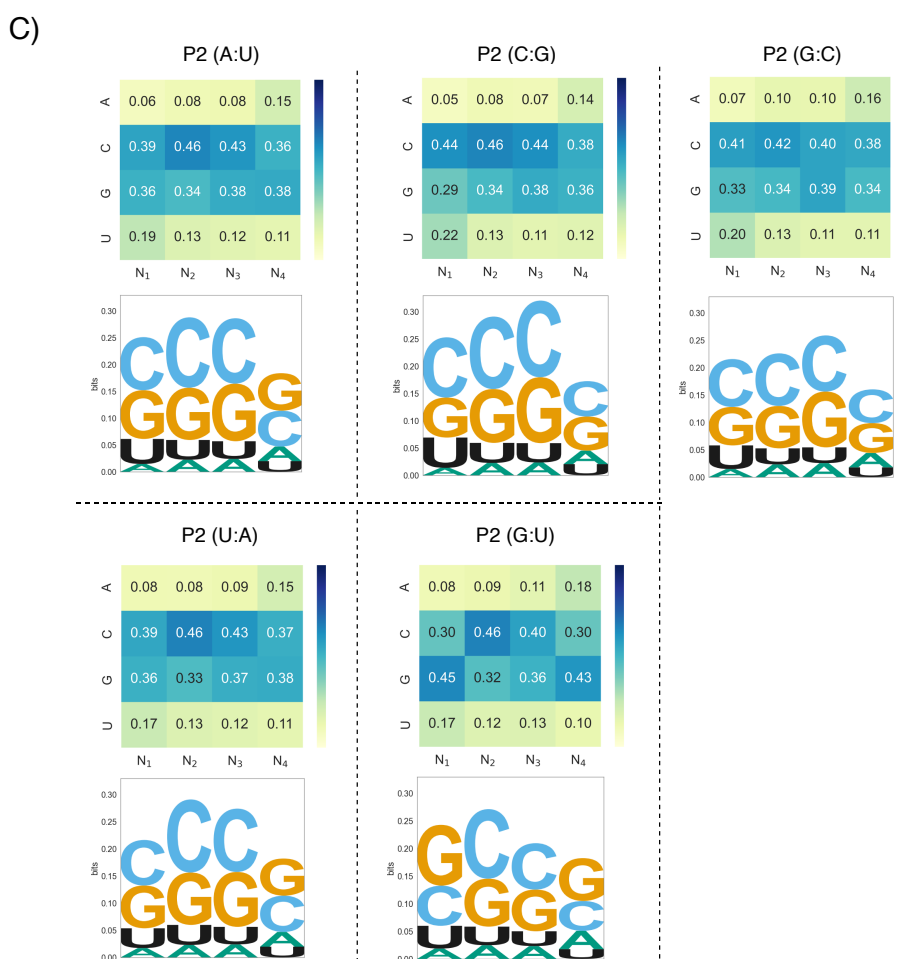
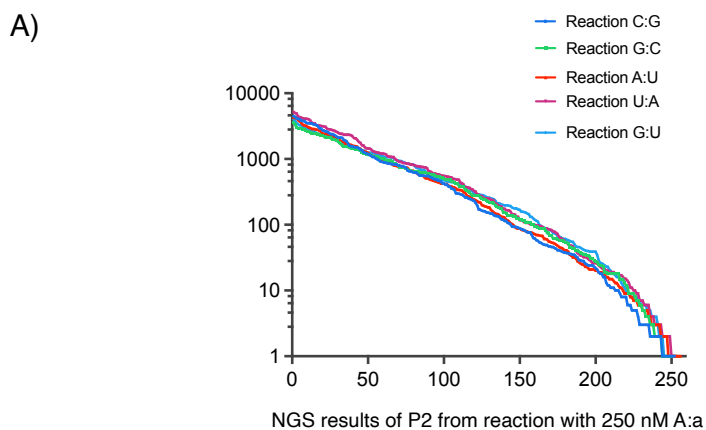
**Figure S6. Deconvolution of splint ligation and loop-closing ligation using four-strand reactions.** Reactions were performed using concentrations of 5 nM or 250 nM for each duplex A:a and B:b. Displayed is a representative gel result from reactions containing 250 nM of each duplex A:a and B:b. Uncropped image can be found in the end of this document.



**Figure S7. Profiles of product P1(A-a) from loop-closing ligation.** A) and B) Product P1 displays logarithmic decrease of sequencing reads relative to their ranking number. C) Heat maps and sequence consensus logos for P1 (information content from 0 to 2 bits), derived from the six reactions at 5 nM featuring different closing base-pairs, show distinct patterns compared to the starting distribution presented in Figure S4.



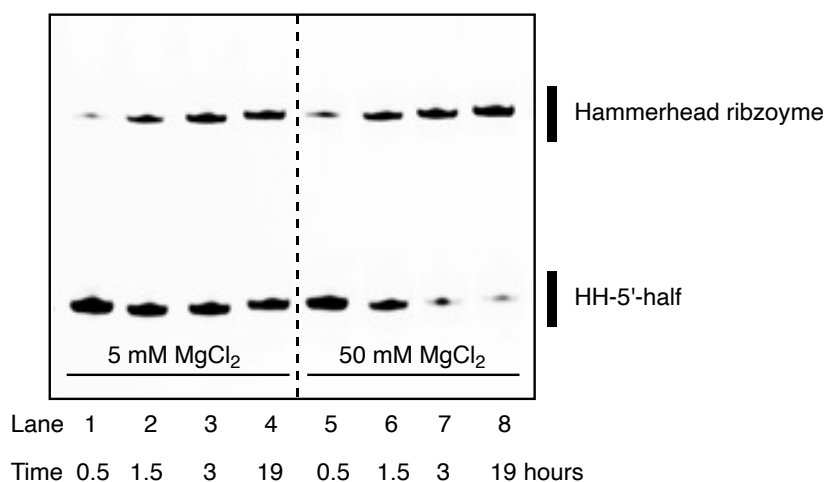
**Figure S8. Changes of probability distributions from starting strand C-N4 to products P1 (loop-closing ligation) and P2 (splint ligation).** Representative results of reaction C:G at 5 nM as shown in Figure S6. The probability distribution of P1, resulting from loop-closing ligation, is significantly more varied and rugged compared to that of P2 or the starting strand.



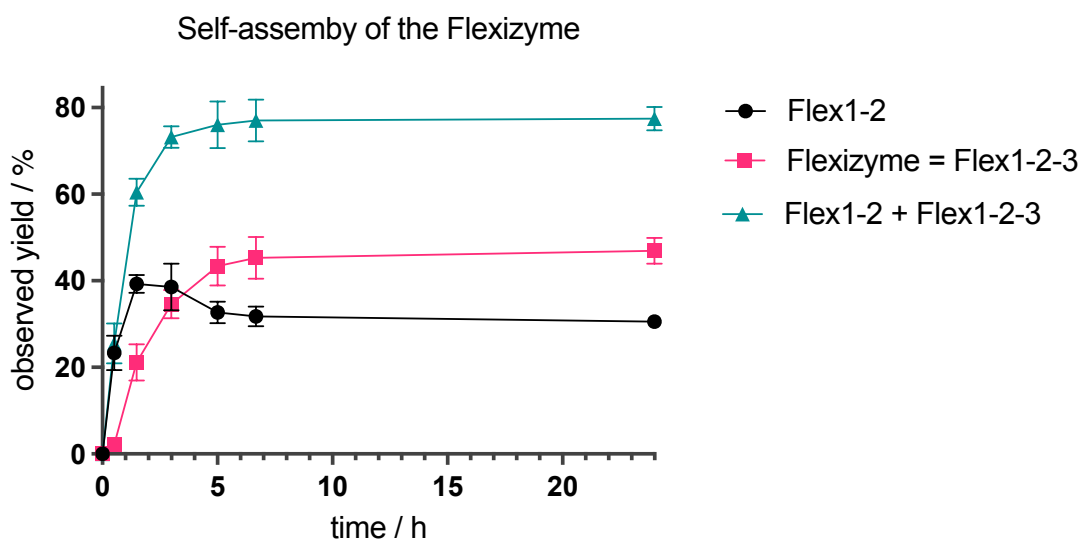
**Figure S9. Profiles of the product P2 from splint ligation (reactions in Figure S5).**

A) Product P2 exhibits a logarithmic decrease in sequencing reads relative to their ranking number. B) Heat maps and sequence consensus logos for P2 (information content from 0 to 2 bits), derived from the six reactions at 250 nM featuring different

closing base-pairs, show patterns that are distinct from those of loop-closing ligation, as demonstrated in Figure S6.

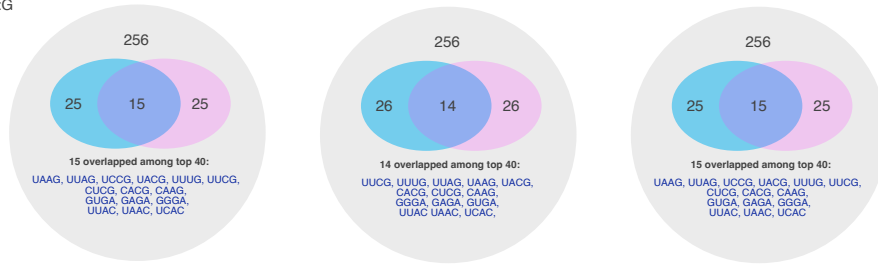


**Figure S10. Representative gel of self-assembling the Hammerhead ribozyme via one loop-closing ligation (See also Figure 5).** Uncropped image can be found in the end of this document.

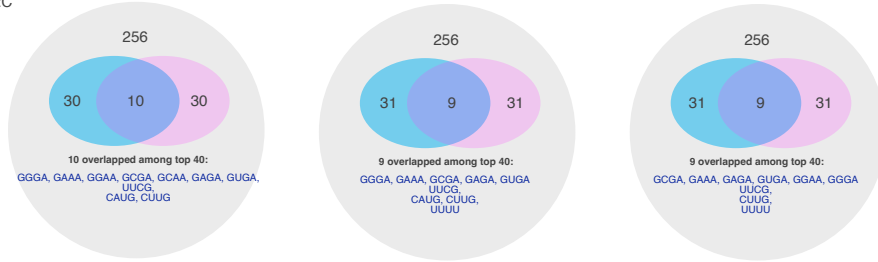


**Figure S11. Time course of the self-assembly of the Flexizyme by two simultaneous loop-closing ligations (See also Figure 5).** The assembly process resulted in a 77% observed yield for one loop (Flex1-2 + Flex1-2-3), while the closure of the other loop was inferred to occur with a 58% yield, calculated as the ratio (Flex1-2-3)/(Flex1-2 + Flex1-2-3).

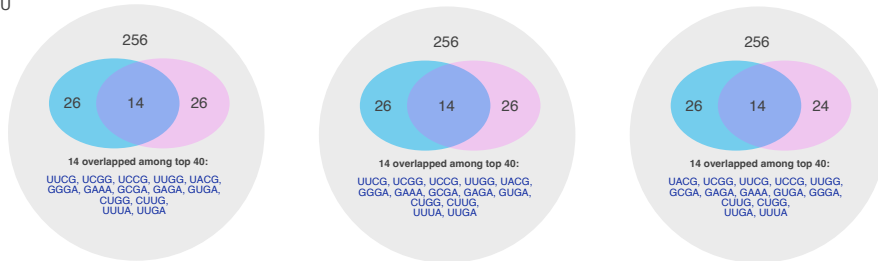
X:Y = C:G



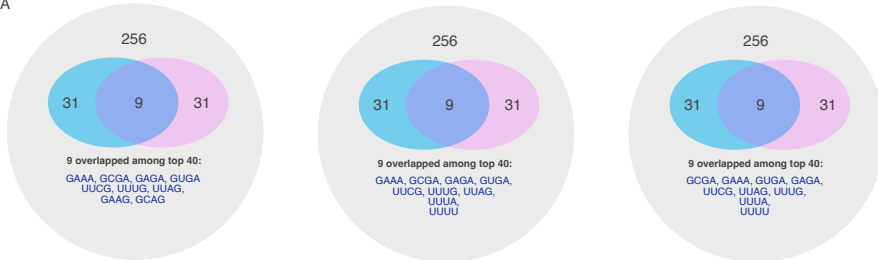
X:Y = G:C



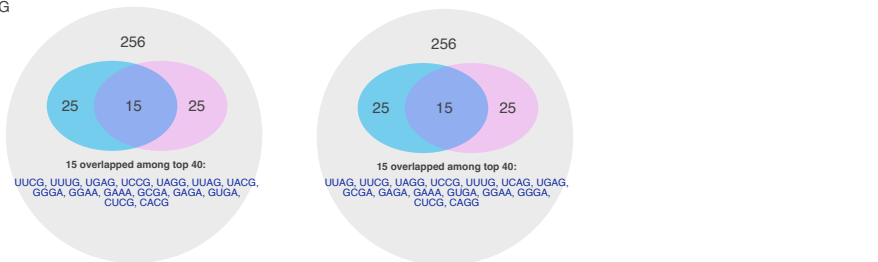
X:Y = A:U



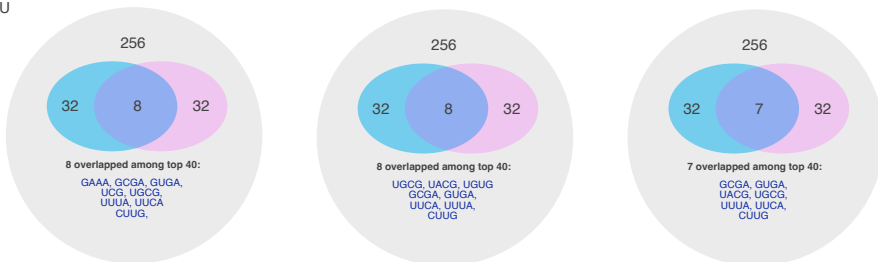
X:Y = U:A



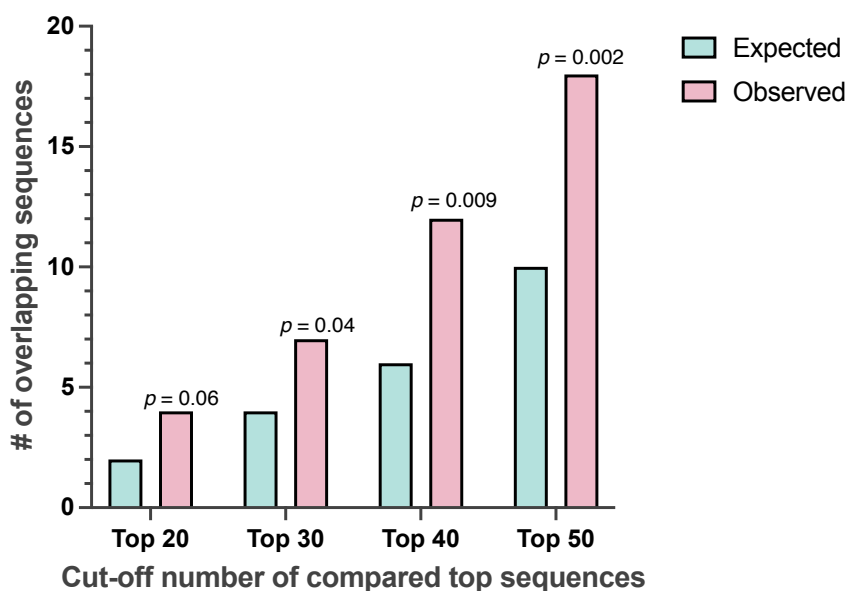
X:Y = U:G



X:Y = G:U

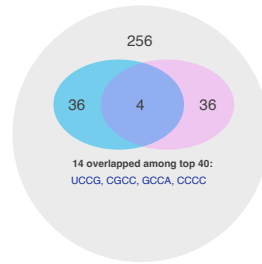
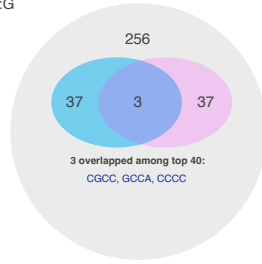


**Figure S12. Overlap of top 40 sequences from loop-closing ligation with top 40 sequences in biological tetraloops.** The data includes results from three independent experiments for each of the six reactions, with the exception of the U:G reaction.

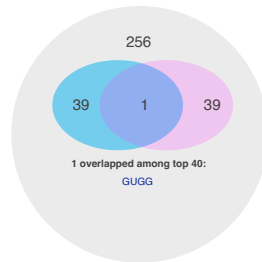
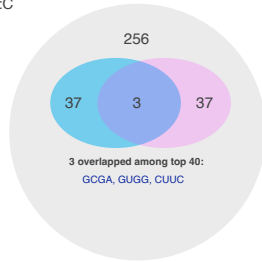


**Figure S13. Over-representation of overlapping sequences across different cut-off number.** Hypergeometric p-value indicating over-representation is shown for each cut-off number. Regardless of the cut-off numbers used (20, 30, 40, or 50 respectively) to define the top sequences, across all comparisons, the overlapping sequences showed approximately a 2-fold over-representation compared to what would be expected by chance.

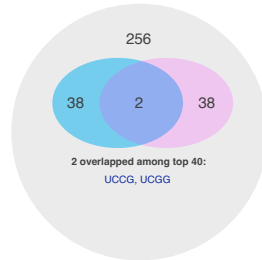
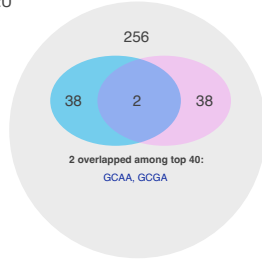
X:Y = C:G



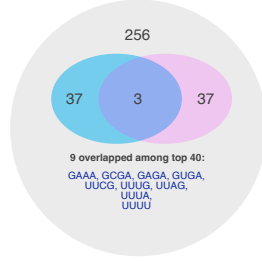
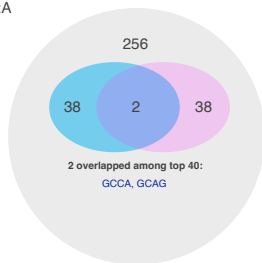
X:Y = G:C



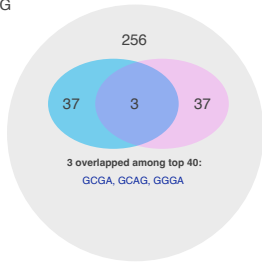
X:Y = A:U



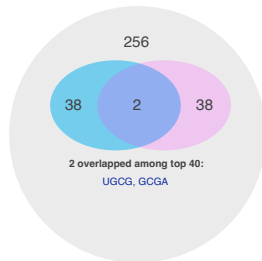
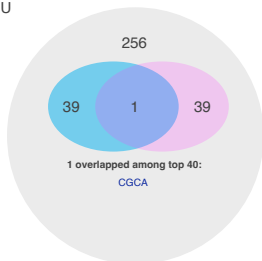
X:Y = U:A



X:Y = U:G



X:Y = G:U



**Figure S14. Overlap of top 40 sequences from splint ligation with top 40 sequences in biological tetraloops.** Two independent experiments for each of the six reactions, except reaction U:G. The data includes results from two independent experiments for each of the six reactions, with the exception of the U:G reaction.

**Table S1. RNA/DNA oligonucleotides used in this work.**

Oligo name	Sequence information	Resource	Notes
A (A-N4)	5'-FAM-GUUCAGAGUUCUACAGUCCGACGAUC <u>UACA</u> GUCAAGUCGUG <u>ANNNN</u> -3'	In-house	RNA, 46nt, N is randomized
A (C-N4)	5'-FAM-GUUCAGAGUUCUACAGUCCGACGAUC <u>UCGA</u> GUCAAGUCGUG <u>CNNNN</u> -3'	In-house	RNA, 46nt, N is randomized
A (G-N4)	5'-FAM-GUUCAGAGUUCUACAGUCCGACGAUC <u>ACAA</u> GUCAAGUCGUG <u>GNNNN</u> -3'	In-house	RNA, 46nt, N is randomized
A (U-N4)	5'-FAM-GUUCAGAGUUCUACAGUCCGACGAUC <u>AGUA</u> GUCAAGUCGUG <u>UNNNN</u> -3'	In-house	RNA, 46nt, N is randomized
a (A-N4)	5'-p- <u>ACACGACUUGAC</u> <u>ACAU</u> AGAUCGGAAGACCACACGUCU-3'	In-house	RNA, 37nt
a (C-N4)	5'-p- <u>CCACGACUUGAC</u> <u>AUCA</u> AGAUCGGAAGACCACACGUCU-3'	In-house	RNA, 37nt
a (G-N4)	5'-p- <u>GCACGACUUGAC</u> <u>AAUA</u> AGAUCGGAAGACCACACGUCU-3'	In-house	RNA, 37nt
a (U-N4)	5'-p- <u>UCACGACUUGAC</u> <u>CUAC</u> AGAUCGGAAGACCACACGUCU-3'	In-house	RNA, 37nt
B	5'-FAM-GUUCAGAGUUCUACAGUCCGACGAUC <u>UAUA</u> CGUAAGCAGCG <u>ANNNN</u> -3'	In-house	RNA, 46nt, N is randomized
b	5'-p- <u>UCGCUGCUUACG</u> <u>AUAA</u> AGAUCGGAAGACCACACGUCU-3'	In-house	RNA, 37nt
a'	5'-p- <u>GCACGACTTGAC</u> <u>GCAA</u> AAGATCGGAAGACCACACGTCT(dd)-3'	IDT	DNA, 38nt, dideoxy modification
Blocker-1	5'-GATCGTCGGACTGTAGAACTCTGAAC-3'	IDT	DNA, 26nt
Blocker-2	5'-AGACGTGTGGTCTTCCGATCT-3'	IDT	DNA, 21nt

RT primer	5'-AGACGTGTGGTCTTCCGATCT-3'	IDT	DNA, 21nt, same as blocker-2
Primer_P5	5'-AATGATACGGCGACCACCGAGATCTACACGTTTCAGAGTTCTACAGTCCG-s-A-3'	IDT	DNA, 50nt, For indexed PCR, -s- indicates phosphorothioate bond
Primer_P7	5'-CAAGCAGAAGACGGCATAACGAGATNNNNNNGTACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3'	NEB	DNA, 64nt, For indexed PCR, -s- indicates phosphorothioate bond, NEB #E7335S/L
Test-donor	5'-p-GCACGAGU-3'	In-house	RNA, 8nt
Test-GCGA	5'-FAM-UUACUCGUGCGCGA-3'	IDT	RNA, 14nt
Test-GAAA	5'-FAM-UUACUCGUGCGAAA-3'	IDT	RNA, 14nt, 1 <sup>st</sup> tset GAAA
Test-UCGC	5'-FAM-UUACUCGUGCUCGC-3'	IDT	RNA, 14nt
Test-UGGC	5'-FAM-UUACUCGUGCUGGC-3'	IDT	RNA, 14nt
Test-CCUG	5'-FAM-UUACUCGUGCCCUG-3'	IDT	RNA, 14nt
Test-CAAG	5'-FAM-UUACUCGUGCCAAG-3'	IDT	RNA, 14nt
Test-CCAG	5'-FAM-UUACUCGUGCCCAG-3'	IDT	RNA, 14nt
Test-AAAU	5'-FAM-UUACUCGUGCAAAU-3'	IDT	RNA, 14nt

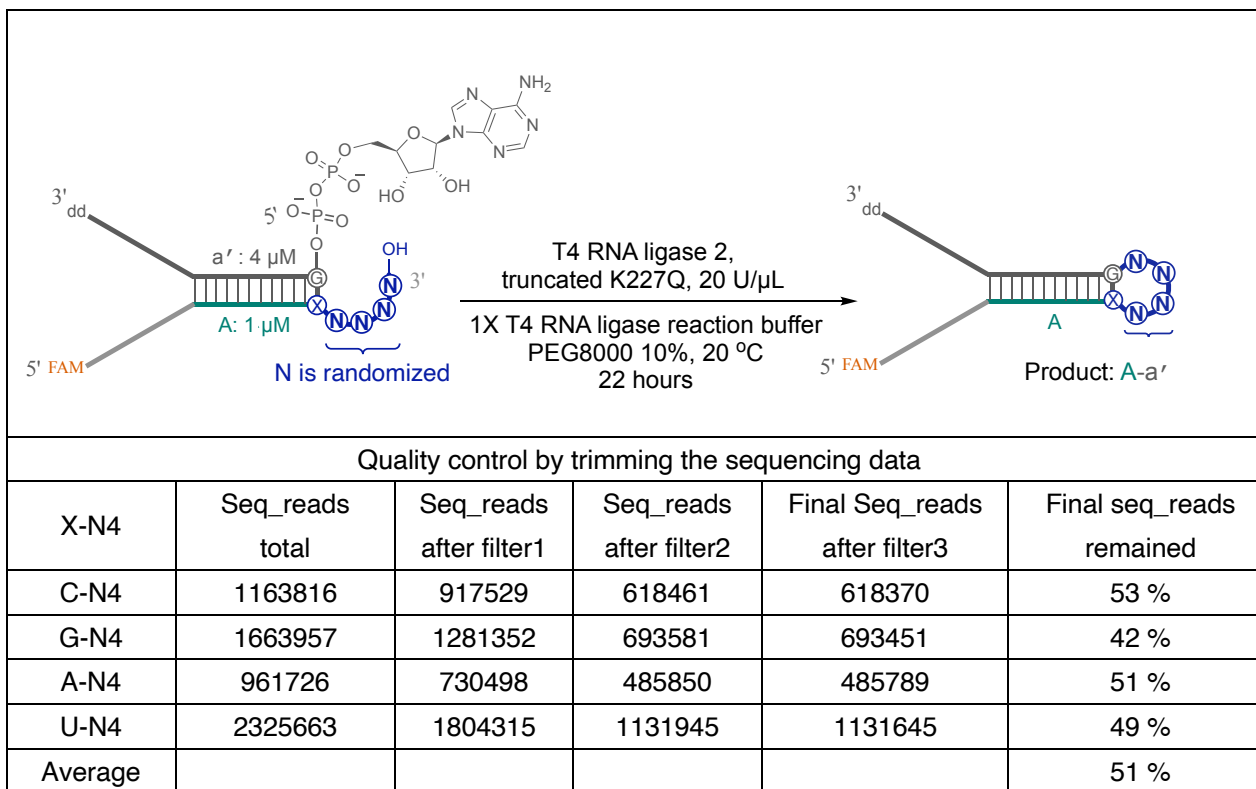
Test-AAGC	5'-FAM-UU <u>ACUCGUGCA</u> AAGC-3'	IDT	RNA, 14nt
Test-GCCC	5'-FAM-UU <u>ACUCGUGCG</u> CCC-3'	IDT	RNA, 14nt
Test-GACU	5'-FAM-UU <u>ACUCGUGCG</u> ACU-3'	IDT	RNA, 14nt
Test-AACC	5'-FAM-UU <u>ACUCGUGCA</u> ACC-3'	IDT	RNA, 14nt
Test-UCCA	5'-FAM-UU <u>ACUCGUGCU</u> CCA-3'	IDT	RNA, 14nt
Test-UUGG	5'-FAM-UU <u>ACUCGUGCU</u> UGG-3'	IDT	RNA, 14nt
Test-UGGU	5'-FAM-UU <u>ACUCGUGCU</u> GGU-3'	IDT	RNA, 14nt
Test-UUCG	5'-FAM-UU <u>ACUCGUGCU</u> UCG-3'	IDT	RNA, 14nt
Test-UACG	5'-FAM-UU <u>ACUCGUGCU</u> ACG-3'	IDT	RNA, 14nt
Test-UUUA	5'-FAM-UU <u>ACUCGUGCU</u> UUA-3'	IDT	RNA, 14nt
Test-UAAC	5'-FAM-UU <u>ACUCGUGCU</u> AAC-3'	IDT	RNA, 14nt
Test-UUAG	5'-FAM-UU <u>ACUCGUGCU</u> UAG-3'	IDT	RNA, 14nt
Test-GAAA	5'-FAM-UU <u>ACUCGUGCG</u> AAA-3'	IDT	RNA, 14nt, 2 <sup>nd</sup> test GAAA
Test-UGGG	5'-FAM-UU <u>ACUCGUGCU</u> GGG-3	IDT	RNA, 14nt
Test-UCGG	5'-FAM-UU <u>ACUCGUGCU</u> CGG-3	IDT	RNA, 14nt
Test-UGCG	5'-FAM-UU <u>ACUCGUGCU</u> GCG-3	IDT	RNA, 14nt

Test-UCUG	5'-FAM-UU <u>ACUCGUGC</u> UCUG-3'	IDT	RNA, 14nt
Test-UCCG	5'-FAM-UU <u>ACUCGUGC</u> UCCG-3'	IDT	RNA, 14nt, 1 <sup>st</sup> test UCCG
Test-GGGA	5'-FAM-A <u>UCGUGCG</u> GGA-3'	IDT	RNA, 11nt
Test-GAGA	5'-FAM-A <u>UCGUGCG</u> GAGA-3'	IDT	RNA, 11nt
Test-GUGA	5'-FAM-A <u>UCGUGCG</u> GUGA-3'	IDT	RNA, 11nt
Test-CAGG	5'-FAM-A <u>UCGUGCC</u> CAGG-3'	IDT	RNA, 11nt
Test-CUGG	5'-FAM-A <u>UCGUGCC</u> CUGG-3'	IDT	RNA, 11nt
Test-CUCG	5'-FAM-A <u>UCGUGCC</u> CUCG-3'	IDT	RNA, 11nt
Test-UUGC	5'-FAM-A <u>UCGUGC</u> UUGC-3'	IDT	RNA, 11nt
Test-UUUG	5'-FAM-A <u>UCGUGC</u> UUUG-3'	IDT	RNA, 11nt
Test-UCCG	5'-FAM-A <u>UCGUGC</u> UCCG-3'	IDT	RNA, 11nt, 2 <sup>nd</sup> test UCCG
Test-UUCA	5'-FAM-A <u>UCGUGC</u> UUCA-3'	IDT	RNA, 11nt
HH-5'-half	5'-FAM-CCUGUCUGAUGAG <u>UCCU</u> UCG-3'	IDT	RNA, 20nt, for assembly of the Hammerhead ribozyme
HH-3'-half	5'-p- <u>GGACGAAACCGU</u> -3'	IDT	RNA, 12nt, for assembly of the Hammerhead ribozyme

Flex1	5'-FAM- <u>GGACCUUCG</u> -3'	In-house	RNA, 9nt, for assembly of the Flexizyme
Flex2	5'-p- <u>GGUCCCGCAUCCCAUCUUCG</u> -3'	In-house	RNA, 20nt, for assembly of the Flexizyme
Flex3	5'-p- <u>GAUGGUACAUGGCGUUAGGU</u> -3'	In-house	RNA, 20nt, for assembly of the Flexizyme

1

**Table S2. Prior distribution of randomized NNNN overhang by RNA deep sequencing.** Frequency range is calculated based on the difference in reads between the most and least frequent sequences.



Detailed sequencing results ranked by their decreasing reads

rank order	X=C			X=G			X=A			X=U		
	NNNN	Reads	$\alpha_i$	NNNN	Reads	$\alpha_i$	NNNN	Reads	$\alpha_i$	NNNN	Reads	$\alpha_i$
1	AAGC	6223	2.586	AAAC	10741	3.968	AAGC	4356	2.304	AAGC	11563	2.611
2	AAAC	5979	2.483	UAAC	8416	3.098	AGGC	4316	2.278	AGGC	10736	2.432
3	AGGC	5068	2.099	AAGC	8384	3.098	GUAC	4012	2.125	AAAC	9601	2.176
4	AUAC	4633	1.920	AAAA	7955	2.944	AAAC	3919	2.074	UAGC	9402	2.125
5	GUAC	4627	1.920	GAAC	7403	2.739	UAGC	3895	2.048	GUAC	9018	2.048
6	AGAC	4623	1.920	AGAC	7185	2.662	AGUA	3809	1.997	UAAC	8859	1.997
7	UAAC	4618	1.920	AAAG	7017	2.586	UAAC	3669	1.946	AUAC	8593	1.946
8	AAAG	4562	1.894	AUAC	6999	2.586	AUAC	3489	1.843	AGUA	8419	1.894
9	CAAC	4556	1.894	CAAC	6988	2.586	AUGC	3434	1.818	GAGC	8291	1.869
10	AAUC	4504	1.869	ACAC	6827	2.509	AGAC	3367	1.766	AGAC	8238	1.869
11	CAGC	4430	1.843	UAAA	6268	2.304	GAGC	3309	1.741	GGGC	7946	1.792
12	GCAC	4412	1.818	GUAC	6094	2.253	GAAC	3290	1.741	AAUC	7892	1.792
13	GAAC	4395	1.818	AAUC	6009	2.227	GGUA	3276	1.715	CAGC	7891	1.792
14	ACAC	4377	1.818	GCAC	6003	2.227	AAUC	3273	1.715	AUGC	7883	1.792
15	GAGC	4351	1.792	AACA	5875	2.176	AAAG	3198	1.690	GAAC	7853	1.766
16	AAGG	4280	1.766	AAGA	5852	2.150	CAGC	3176	1.664	GGAC	7682	1.741
17	UAGC	4213	1.741	UAGC	5742	2.125	GUAA	3168	1.664	GGUA	7439	1.690
18	AAAA	4195	1.741	AGGC	5732	2.125	GGGC	3140	1.664	GCAC	7377	1.664
19	UAAG	4030	1.664	AACC	5676	2.099	AAGA	3133	1.638	ACAC	7344	1.664

20	AGUA	3911	1.613	AAAU	5668	2.099	AGUC	3105	1.638	CAAC	7243	1.638
21	AACA	3847	1.587	AUAA	5528	2.048	AAAA	3098	1.638	AGUC	7147	1.613
22	AAUA	3843	1.587	GAAA	5471	2.022	AAGG	3080	1.613	AAAA	7125	1.613
23	AUAG	3813	1.587	AAUA	5376	1.997	AAUA	3071	1.613	GUAA	7046	1.587
24	AUGC	3808	1.587	AGAA	5231	1.920	UGGC	2968	1.562	AGCC	7044	1.587
25	AAGA	3807	1.587	UAAG	5028	1.869	GGAC	2959	1.562	UGGC	7022	1.587
26	AACC	3783	1.562	GAGC	4984	1.843	AUAA	2919	1.536	AAGA	6901	1.562
27	AUAA	3767	1.562	CAAA	4893	1.818	UAUC	2894	1.536	AAGG	6898	1.562
28	GUAA	3744	1.562	GGAC	4891	1.818	UAAA	2887	1.510	UAAA	6886	1.562
29	GGAC	3693	1.536	GAAG	4847	1.792	UGUA	2885	1.510	UAUC	6820	1.536
30	AGUC	3549	1.459	AAGG	4843	1.792	UUAC	2871	1.510	UAUA	6761	1.536
31	GGGC	3544	1.459	CAGC	4834	1.792	ACAC	2822	1.485	UAGA	6673	1.510
32	GCAA	3542	1.459	ACAA	4787	1.766	GCAC	2809	1.485	AGCA	6667	1.510
33	GAAA	3539	1.459	AGUA	4718	1.741	UAGA	2786	1.459	AACA	6636	1.510
34	GUAG	3525	1.459	AUAG	4681	1.741	UAUA	2785	1.459	AUAA	6607	1.485
35	GAAG	3521	1.459	GUAA	4637	1.715	AGCC	2774	1.459	AAUA	6607	1.485
36	AGCC	3499	1.459	GCAA	4627	1.715	AUAG	2766	1.459	ACGC	6557	1.485
37	AAUG	3462	1.434	AGCC	4580	1.690	AGCA	2766	1.459	UUAC	6554	1.485
38	ACGC	3431	1.408	UAGA	4490	1.664	GUGC	2765	1.459	GGUC	6527	1.485
39	GAUC	3427	1.408	UAUC	4329	1.587	AGAA	2737	1.434	AAAG	6482	1.459
40	UAUA	3421	1.408	AGCA	4304	1.587	AGGA	2732	1.434	AGGA	6468	1.459
41	AGCA	3413	1.408	CCAC	4282	1.587	CAAC	2728	1.434	GCGC	6453	1.459
42	UAAA	3412	1.408	UUAC	4251	1.562	GUAG	2704	1.434	UGUA	6438	1.459
43	AAAU	3400	1.408	ACAG	4220	1.562	AACA	2682	1.408	AAGU	6336	1.434

44	AGAA	3378	1.408	AGAG	4216	1.562	GCGC	2647	1.382	GUGC	6319	1.434
45	GCGC	3318	1.382	UAAU	4209	1.562	AAUG	2613	1.382	AACC	6302	1.434
46	CAUC	3313	1.382	AUGC	4185	1.536	ACGC	2612	1.382	GAUC	6246	1.408
47	AGAG	3311	1.382	AGUC	4108	1.510	GGUC	2605	1.382	AGAA	6231	1.408
48	AAGU	3289	1.357	AAGU	4053	1.485	UAAG	2581	1.357	GGCC	6223	1.408
49	CUAC	3288	1.357	CAAG	4053	1.485	GAAG	2571	1.357	UACA	6124	1.382
50	UUAC	3286	1.357	AGGA	4034	1.485	GGCA	2550	1.331	UAAG	5954	1.357
51	GGUA	3273	1.357	UACA	4029	1.485	GGCC	2550	1.331	GCAA	5895	1.331
52	AGGA	3218	1.331	UAUA	4011	1.485	AAGU	2526	1.331	GGCA	5862	1.331
53	CCAC	3198	1.331	UGAC	3989	1.485	UAGG	2514	1.331	GAAA	5852	1.331
54	ACAG	3197	1.331	UCAC	3957	1.459	UGAC	2509	1.331	UGAC	5836	1.331
55	UAGA	3176	1.306	UACC	3944	1.459	AUUC	2485	1.306	CAUC	5742	1.306
56	CAAG	3161	1.306	GAUC	3821	1.408	GAAA	2475	1.306	GUAG	5698	1.280
57	UAUC	3120	1.280	AAUG	3820	1.408	AACC	2473	1.306	UUAA	5639	1.280
58	UACA	3109	1.280	ACGC	3800	1.408	AUUA	2472	1.306	UAGG	5636	1.280
59	UAGG	3086	1.280	GACC	3755	1.382	GAUA	2460	1.306	CGGC	5612	1.280
60	ACAA	3067	1.280	GACA	3722	1.382	UUAA	2405	1.280	GAUA	5564	1.254
61	GGCC	3040	1.254	GAAU	3719	1.382	AGAG	2401	1.254	AUUC	5564	1.254
62	GCCA	3035	1.254	AGAU	3690	1.357	AUGG	2388	1.254	AAAU	5531	1.254
63	GCAG	3017	1.254	GUAG	3662	1.357	GGAA	2374	1.254	AUUA	5529	1.254
64	GGCA	2986	1.229	AUAU	3662	1.357	AGGG	2369	1.254	GGAA	5522	1.254
65	UGUA	2968	1.229	GGAA	3643	1.357	UUGC	2365	1.254	AUAG	5459	1.229
66	GCUC	2966	1.229	GAGA	3633	1.331	GCAA	2362	1.254	AGGG	5416	1.229
67	CAAA	2958	1.229	CAUC	3585	1.331	GUUA	2356	1.229	AGGU	5402	1.229

68	GACC	2945	1.229	GAUA	3535	1.306	GAUC	2356	1.229	AAUG	5382	1.229
69	GAUA	2901	1.203	CAAU	3497	1.280	GAGG	2312	1.229	GCUC	5376	1.229
70	GACA	2896	1.203	ACCA	3480	1.280	AUGA	2309	1.229	UACC	5345	1.203
71	CGGC	2892	1.203	CGAC	3426	1.254	UACA	2308	1.229	UUGC	5344	1.203
72	GAGG	2887	1.203	UUAA	3416	1.254	GUAU	2305	1.203	GUAU	5341	1.203
73	AUAU	2879	1.203	GCAG	3393	1.254	GAGA	2296	1.203	GAGA	5327	1.203
74	CACC	2877	1.203	CACC	3389	1.254	AAAU	2279	1.203	UAGU	5316	1.203
75	AUUA	2858	1.178	ACAU	3368	1.254	AGUG	2240	1.178	GCCA	5289	1.203
76	AGGG	2849	1.178	CUAC	3366	1.254	GCCA	2235	1.178	ACAA	5266	1.203
77	AUGG	2839	1.178	CACA	3278	1.203	GUUC	2229	1.178	CUAC	5260	1.178
78	UGGC	2839	1.178	CAGA	3207	1.178	CAUC	2223	1.178	AUGA	5226	1.178
79	CGAC	2812	1.152	GCCA	3188	1.178	AGGU	2209	1.152	GUUA	5215	1.178
80	GGAA	2810	1.152	GGUA	3185	1.178	GGGA	2203	1.152	UCAC	5210	1.178
81	UUAG	2808	1.152	ACUC	3170	1.178	GCUC	2198	1.152	GACA	5170	1.178
82	ACUC	2790	1.152	UGAA	3169	1.178	AUAU	2191	1.152	AGAG	5151	1.178
83	UAAU	2787	1.152	AUUA	3119	1.152	ACUC	2171	1.152	UGGA	5131	1.152
84	GAGA	2772	1.152	GGCC	3075	1.126	UAUG	2167	1.152	GAAG	5121	1.152
85	UAUG	2768	1.152	AUUC	3064	1.126	CGGC	2163	1.152	GACC	5121	1.152
86	UUAA	2761	1.152	AAUU	3062	1.126	UACC	2144	1.126	GGGA	5113	1.152
87	CACA	2756	1.152	AUGA	3043	1.126	UGUC	2140	1.126	GAGG	5093	1.152
88	GGUC	2754	1.152	GAGG	3021	1.126	CUAC	2136	1.126	ACUC	5069	1.152
89	GUAU	2736	1.126	ACCC	3020	1.126	ACAG	2133	1.126	CCAC	5034	1.126
90	AACG	2731	1.126	CAUA	2996	1.101	UAGU	2117	1.126	UAAU	5031	1.126
91	GUGC	2728	1.126	GGCA	2970	1.101	UUAG	2110	1.101	AUAU	5017	1.126

92	UAGU	2708	1.126	GCGC	2964	1.101	UGGA	2106	1.101	GUUC	5017	1.126
93	UGAC	2696	1.126	AACG	2951	1.101	UAAU	2092	1.101	AUGG	4995	1.126
94	UACC	2691	1.126	UGGC	2934	1.075	GACA	2090	1.101	CAAA	4983	1.126
95	UCAC	2674	1.101	UAGG	2932	1.075	ACAA	2077	1.101	AUCA	4815	1.101
96	GCCC	2654	1.101	GGGC	2926	1.075	UUUA	2072	1.101	GCUA	4783	1.075
97	CAGG	2650	1.101	GUAU	2900	1.075	GGAG	2058	1.075	CACA	4777	1.075
98	ACCA	2648	1.101	CUAA	2864	1.050	GCUA	2058	1.075	ACCA	4751	1.075
99	CAUA	2645	1.101	AUCA	2856	1.050	GACC	2031	1.075	UUUA	4728	1.075
100	AGUG	2644	1.101	AUCC	2839	1.050	UGAA	2025	1.075	CAGA	4718	1.075
101	GGAG	2639	1.101	CCAA	2820	1.050	UCAC	2024	1.075	AGAU	4717	1.075
102	CAGA	2635	1.101	UAGU	2815	1.050	CAAG	2017	1.075	UAUG	4683	1.050
103	GAAU	2629	1.101	AGGG	2760	1.024	AUUG	2009	1.050	UGUC	4679	1.050
104	AAUU	2614	1.075	GCAU	2759	1.024	GCAG	2003	1.050	CAUA	4653	1.050
105	AGAU	2613	1.075	GGAG	2749	1.024	AGAU	2000	1.050	CGAC	4650	1.050
106	AUUC	2585	1.075	GCCC	2712	0.998	CAUA	1998	1.050	UGAA	4650	1.050
107	CAAU	2579	1.075	UUAG	2638	0.973	CAAA	1976	1.050	UGCA	4644	1.050
108	AGGU	2537	1.050	GGUC	2631	0.973	AUCA	1966	1.024	ACAG	4546	1.024
109	AUGA	2530	1.050	UCAA	2619	0.973	CCAC	1962	1.024	CACC	4487	1.024
110	GAUG	2518	1.050	UGUA	2581	0.947	ACCA	1939	1.024	GAAU	4485	1.024
111	ACCC	2511	1.050	ACUA	2574	0.947	AUGU	1938	1.024	UGCC	4480	1.024
112	AUCA	2503	1.024	UAUG	2519	0.922	GAUG	1936	1.024	AAUU	4461	0.998
113	AUUG	2432	0.998	CAGG	2515	0.922	UGCA	1931	1.024	GGAG	4444	0.998
114	GCUA	2422	0.998	AGGU	2505	0.922	GUGA	1931	1.024	UUAG	4434	0.998
115	ACAU	2419	0.998	GGAU	2468	0.922	UUUC	1928	1.024	GCAG	4431	0.998

116	AGCG	2401	0.998	ACGA	2454	0.896	AAUU	1924	1.024	CAAG	4392	0.998
117	GUUA	2371	0.973	GCUC	2428	0.896	CAGA	1916	0.998	AGUG	4389	0.998
118	CGCC	2367	0.973	GAUG	2426	0.896	CAGG	1899	0.998	AUGU	4380	0.998
119	CUAG	2365	0.973	AUGG	2405	0.896	UGCC	1892	0.998	GUGA	4369	0.998
120	CAGU	2359	0.973	AACU	2396	0.896	CGUA	1884	0.998	UCGC	4358	0.998
121	GCAU	2353	0.973	CGAA	2349	0.870	CUGC	1882	0.998	GAGU	4354	0.973
122	GUUC	2351	0.973	AGUG	2347	0.870	GAAU	1867	0.973	GCCC	4344	0.973
123	CAUG	2334	0.973	GAGU	2337	0.870	AGUU	1854	0.973	GCGA	4272	0.973
124	UGAA	2317	0.947	CGGC	2320	0.845	UCGC	1851	0.973	CGUA	4242	0.947
125	CUAA	2305	0.947	GUGC	2293	0.845	AGCG	1850	0.973	GUCA	4240	0.947
126	UUGC	2278	0.947	UGGA	2265	0.845	CGAC	1822	0.973	AUCC	4225	0.947
127	GCGA	2260	0.947	GCUA	2260	0.845	GAGU	1821	0.947	AGUU	4212	0.947
128	UGGA	2256	0.922	GCGA	2247	0.819	UUGA	1804	0.947	GGAU	4193	0.947
129	GGGA	2246	0.922	AGCG	2226	0.819	GGUG	1802	0.947	UUUC	4168	0.947
130	AUGU	2217	0.922	CAGU	2220	0.819	UUAU	1776	0.947	CAGG	4164	0.947
131	UUUA	2209	0.922	UGCC	2205	0.819	GCCC	1767	0.922	ACCC	4160	0.947
132	UGCA	2208	0.922	GGGA	2200	0.819	GUCA	1767	0.922	GAUG	4138	0.947
133	GAGU	2198	0.922	UUAU	2182	0.794	GCGA	1757	0.922	CAAU	4126	0.922
134	ACUA	2194	0.896	CCAG	2147	0.794	CACA	1753	0.922	UAUU	4119	0.922
135	AUCC	2190	0.896	UUGC	2137	0.794	GUGG	1742	0.922	ACUA	4106	0.922
136	CGUA	2169	0.896	GUUA	2117	0.794	AUCC	1735	0.922	UUGA	4102	0.922
137	GUCA	2162	0.896	UCAG	2102	0.768	ACUA	1712	0.896	ACAU	4095	0.922
138	UACG	2159	0.896	UGAG	2084	0.768	ACCC	1701	0.896	CAGU	4072	0.922
139	CUGC	2155	0.896	CGCC	2084	0.768	UGAG	1700	0.896	UUAU	4066	0.922

140	CCGC	2152	0.896	UGCA	2079	0.768	CACC	1699	0.896	CUGC	4031	0.922
141	CGCA	2141	0.896	AGCU	2069	0.768	CUAA	1687	0.896	UCAA	3997	0.896
142	UGAG	2132	0.870	UAUU	2059	0.768	UAUU	1682	0.896	AGCG	3993	0.896
143	UCAG	2123	0.870	CUAG	2051	0.768	CAGU	1676	0.896	CUAA	3974	0.896
144	UAUU	2119	0.870	AUUG	2016	0.742	GGAU	1668	0.870	CGCC	3929	0.896
145	CCAG	2116	0.870	GUUC	2012	0.742	CAAU	1667	0.870	GGGU	3876	0.870
146	UUAU	2083	0.870	ACGG	2008	0.742	CAUG	1660	0.870	AUUG	3859	0.870
147	CCAA	2080	0.870	CAUG	2005	0.742	AACG	1657	0.870	UGGU	3859	0.870
148	UCAA	2055	0.845	AUGU	2003	0.742	GUUG	1652	0.870	CGUC	3829	0.870
149	ACGG	2052	0.845	GUCA	1998	0.742	ACAU	1646	0.870	GCAU	3801	0.870
150	UGCC	2037	0.845	UCGC	1972	0.717	CCGC	1618	0.845	GUCC	3796	0.870
151	GGAU	2035	0.845	CGCA	1971	0.717	ACGG	1616	0.845	GUGG	3790	0.845
152	GUGG	2035	0.845	AGUU	1964	0.717	CGCC	1615	0.845	AACG	3778	0.845
153	GCGG	2030	0.845	CCAU	1952	0.717	GGGU	1615	0.845	CCGC	3755	0.845
154	CGUC	2021	0.845	UGUC	1948	0.717	UUGG	1610	0.845	GGUU	3723	0.845
155	AGUU	2021	0.845	GAUU	1941	0.717	CUAG	1610	0.845	AGCU	3679	0.845
156	CACG	2012	0.845	UGAU	1919	0.717	AUUU	1601	0.845	GGUG	3651	0.819
157	CGAA	2005	0.819	GACG	1916	0.717	UCAA	1598	0.845	GGGG	3649	0.819
158	GAUU	1984	0.819	CGUA	1915	0.717	GGUU	1594	0.845	ACGA	3630	0.819
159	GGUG	1974	0.819	UACG	1901	0.691	GCGG	1578	0.819	CGCA	3616	0.819
160	AUUU	1969	0.819	GUGA	1891	0.691	UGGU	1572	0.819	GAUU	3601	0.819
161	UCGC	1964	0.819	UUUA	1833	0.666	ACGA	1571	0.819	UGAG	3585	0.819
162	GACG	1959	0.819	CGAG	1822	0.666	CGUC	1558	0.819	UUCA	3583	0.819
163	GUGA	1948	0.819	CUAU	1817	0.666	GCAU	1555	0.819	GGCG	3515	0.794

164	UUGA	1932	0.794	GUCC	1816	0.666	GGGG	1555	0.819	AUUU	3513	0.794
165	GGGG	1925	0.794	CCCA	1813	0.666	GUCC	1546	0.819	CGGA	3463	0.794
166	CAUU	1921	0.794	CCGC	1761	0.640	GGCG	1540	0.819	UGAU	3446	0.768
167	AACU	1907	0.794	CAUU	1759	0.640	UGGG	1538	0.819	UGGG	3437	0.768
168	AGCU	1899	0.794	CGAU	1735	0.640	AGCU	1526	0.794	CAUG	3417	0.768
169	GGCG	1895	0.794	UCAU	1733	0.640	UGUG	1516	0.794	UCUC	3405	0.768
170	CUAU	1885	0.768	UCCA	1716	0.640	GAUU	1504	0.794	GUUG	3375	0.768
171	ACGA	1876	0.768	UUGA	1716	0.640	CGCA	1474	0.768	ACGG	3373	0.768
172	UGUC	1868	0.768	UACU	1694	0.614	UUCA	1457	0.768	GCGG	3337	0.742
173	CGAG	1860	0.768	CGUC	1690	0.614	GUGU	1423	0.742	UUGU	3307	0.742
174	GUCC	1836	0.768	CCCC	1678	0.614	GUUU	1422	0.742	UUGG	3306	0.742
175	UUGG	1818	0.742	AUUU	1667	0.614	UCAG	1412	0.742	GUGU	3305	0.742
176	GUUG	1800	0.742	GACU	1665	0.614	UACG	1411	0.742	CGAA	3299	0.742
177	CCUC	1796	0.742	CACG	1654	0.614	UCUC	1411	0.742	GGCU	3287	0.742
178	UUUC	1793	0.742	CUGC	1634	0.614	UGAU	1403	0.742	AACU	3250	0.742
179	CCCA	1771	0.742	UUUC	1597	0.589	CGAA	1403	0.742	CCAA	3247	0.742
180	CCCC	1759	0.717	ACUG	1583	0.589	GACG	1396	0.742	CAUU	3232	0.742
181	UGAU	1756	0.717	UUCA	1580	0.589	UCUA	1383	0.717	UACG	3205	0.717
182	AUCG	1723	0.717	CGGA	1557	0.563	UUUG	1379	0.717	CUAG	3190	0.717
183	UUCA	1719	0.717	ACGU	1554	0.563	GGCU	1377	0.717	UCCA	3186	0.717
184	GCUG	1703	0.717	UCUC	1547	0.563	CUUA	1374	0.717	UCAG	3152	0.717
185	CUUC	1697	0.691	GCGG	1543	0.563	CUUC	1367	0.717	ACGU	3149	0.717
186	UGGU	1693	0.691	AUCG	1533	0.563	CUAU	1364	0.717	GUUU	3148	0.717
187	CCAU	1688	0.691	CCUC	1477	0.538	CCAA	1349	0.717	CUUA	3141	0.717

188	UGGG	1685	0.691	CUCA	1463	0.538	UUCC	1345	0.717	CCUC	3092	0.691
189	CUCA	1663	0.691	UCCC	1452	0.538	CCAG	1343	0.717	GCGU	2986	0.666
190	UCCA	1662	0.691	CACU	1448	0.538	CGGA	1340	0.717	UGUG	2981	0.666
191	CUUA	1654	0.691	GGCG	1412	0.512	CCUC	1339	0.717	CUUC	2963	0.666
192	GGGU	1654	0.691	UUCC	1345	0.486	ACGU	1336	0.717	GACG	2955	0.666
193	CGGA	1623	0.666	ACCG	1340	0.486	UUGU	1327	0.691	UGUU	2952	0.666
194	UACU	1622	0.666	GGUG	1319	0.486	ACUG	1318	0.691	UCUA	2940	0.666
195	ACUG	1620	0.666	CUUA	1318	0.486	GCUG	1307	0.691	UACU	2932	0.666
196	GGUU	1610	0.666	UCGA	1306	0.486	UCCA	1302	0.691	CUCA	2930	0.666
197	GGCU	1584	0.666	UCUA	1292	0.486	CAUU	1298	0.691	UUCC	2917	0.666
198	GACU	1583	0.666	GGGU	1290	0.486	AACU	1298	0.691	UCGA	2901	0.666
199	GCGU	1562	0.640	GUGG	1284	0.486	AUCG	1271	0.666	CCAG	2896	0.666
200	UCAU	1558	0.640	ACUU	1274	0.461	UGUU	1250	0.666	UCAU	2881	0.640
201	GUGU	1556	0.640	UGGU	1269	0.461	CUGG	1247	0.666	CUAU	2869	0.640
202	UCUC	1554	0.640	GGCU	1263	0.461	CCCA	1247	0.666	UUUG	2849	0.640
203	CGAU	1549	0.640	CUCC	1260	0.461	CUCA	1242	0.666	AUCG	2822	0.640
204	CUGG	1540	0.640	GGGG	1236	0.461	UUUU	1239	0.666	UCCC	2799	0.640
205	ACGU	1529	0.640	AUCU	1226	0.461	GCGU	1237	0.640	CCCA	2791	0.640
206	CUCC	1524	0.640	GCGU	1223	0.461	CGAG	1236	0.640	CGGU	2715	0.614
207	UCUA	1513	0.614	CUGA	1214	0.461	CGGG	1207	0.640	GACU	2714	0.614
208	CGCG	1510	0.614	GGUU	1214	0.461	CACG	1203	0.640	CUGA	2694	0.614
209	UUUG	1503	0.614	CCUA	1211	0.435	CUGA	1178	0.614	ACUG	2694	0.614
210	CGGG	1499	0.614	CUUC	1196	0.435	UGCG	1169	0.614	UUUU	2693	0.614
211	UGUG	1497	0.614	ACCU	1185	0.435	UACU	1163	0.614	CGAG	2633	0.589

212	GCCG	1495	0.614	GCUG	1180	0.435	UCAU	1152	0.614	GCUG	2623	0.589
213	UGCG	1477	0.614	UUGG	1169	0.435	GUCG	1129	0.589	GCUU	2619	0.589
214	ACCG	1474	0.614	GUUG	1159	0.435	ACUU	1125	0.589	CGAU	2604	0.589
215	UUGU	1461	0.614	UGGG	1158	0.435	GACU	1117	0.589	CACG	2590	0.589
216	CACU	1457	0.614	GUUU	1128	0.410	CCAU	1111	0.589	UGCG	2582	0.589
217	GUUU	1421	0.589	GUGU	1090	0.410	UCGA	1109	0.589	CCAU	2561	0.589
218	GUCG	1408	0.589	UUGU	1049	0.384	CGGU	1099	0.589	CGGG	2551	0.589
219	CGUG	1405	0.589	GCUU	1048	0.384	UCCC	1093	0.563	CUCC	2550	0.589
220	UCCC	1401	0.589	CCGA	1047	0.384	UGCU	1088	0.563	CCCC	2518	0.563
221	UUCC	1395	0.589	GCCG	1043	0.384	CGUG	1081	0.563	ACUU	2469	0.563
222	UCGA	1392	0.589	UGUG	1029	0.384	ACCG	1069	0.563	CUGG	2435	0.563
223	ACUU	1392	0.589	GUCG	1001	0.358	CGAU	1067	0.563	UGCU	2397	0.538
224	CGGU	1372	0.563	UGCG	993	0.358	CUUG	1058	0.563	CACU	2366	0.538
225	CUGA	1350	0.563	GCCU	992	0.358	CCCC	1049	0.563	AUCU	2323	0.538
226	GCUU	1349	0.563	CGGG	925	0.333	CUCC	1045	0.563	CCUA	2282	0.512
227	CUUG	1321	0.538	UUUG	925	0.333	CCUA	1032	0.538	GUCG	2255	0.512
228	UCGG	1302	0.538	UGUU	923	0.333	GCUU	1031	0.538	CGUG	2248	0.512
229	AUCU	1294	0.538	CGCG	918	0.333	GCCG	1025	0.538	UCGG	2203	0.486
230	CCUA	1285	0.538	CGCU	910	0.333	UCGG	1019	0.538	UCGU	2146	0.486
231	UGUU	1272	0.538	CGGU	900	0.333	AUCU	1015	0.538	GCCG	2145	0.486
232	UUUU	1232	0.512	UGCU	895	0.333	CGCG	978	0.512	CGCU	2131	0.486
233	CCGA	1208	0.512	CUGG	894	0.333	CACU	974	0.512	GUCU	2126	0.486
234	UGCU	1186	0.486	UCGG	879	0.333	CUGU	958	0.512	CGCG	2110	0.486
235	CGCU	1180	0.486	UUUU	869	0.333	CUUU	942	0.486	GCCU	2108	0.486

236	CCGG	1163	0.486	CGUG	846	0.307	UCGU	906	0.486	CGUU	2088	0.461
237	CUUU	1129	0.461	GUCU	840	0.307	CGUU	899	0.486	CUGU	2085	0.461
238	CGUU	1128	0.461	CGUU	785	0.282	UUCG	894	0.461	ACCU	2079	0.461
239	UUCG	1125	0.461	CUGU	752	0.282	GUCU	889	0.461	ACCG	2036	0.461
240	CUGU	1105	0.461	UCGU	738	0.282	CCGA	888	0.461	CCGA	1978	0.435
241	ACCU	1097	0.461	CUUG	737	0.282	CGCU	878	0.461	CUUG	1942	0.435
242	CCUG	1071	0.435	CUUU	701	0.256	ACCU	864	0.461	CUUU	1907	0.435
243	GCCU	1069	0.435	CCGU	694	0.256	UCUG	864	0.461	UUCG	1860	0.410
244	UCGU	1055	0.435	CCGG	684	0.256	CCGG	853	0.461	UCUG	1764	0.410
245	CUCG	1045	0.435	UUCG	684	0.256	GCCU	838	0.435	CCGU	1674	0.384
246	GUCU	1028	0.435	CUCG	662	0.256	CUCG	776	0.410	UCUU	1651	0.384
247	UCUG	1026	0.435	UCUG	647	0.230	CCGU	774	0.410	CCGG	1650	0.384
248	CCCG	1003	0.410	CCCU	620	0.230	UUCU	771	0.410	UUCU	1639	0.358
249	CCGU	969	0.410	UCUU	618	0.230	UCUU	765	0.410	CUCG	1521	0.333
250	CCUU	878	0.358	UUCU	615	0.230	CCUG	760	0.410	CCUU	1434	0.333
251	UCCG	873	0.358	CCUG	602	0.230	CCUU	672	0.358	CUCU	1433	0.333
252	UCUU	816	0.333	UCCG	580	0.205	CUCU	614	0.333	CCUG	1370	0.307
253	CUCU	812	0.333	CCUU	576	0.205	UCCG	598	0.307	UCCG	1272	0.282
254	UUCU	774	0.333	CCCG	573	0.205	CCCG	583	0.307	CCCU	1260	0.282
255	CCCU	732	0.307	CUCU	554	0.205	CCCU	535	0.282	UCCU	1236	0.282
256	UCCU	712	0.307	UCCU	550	0.205	UCCU	521	0.282	CCCG	1228	0.282
Frequency range		9		20			8			9		

**Table S3. Global overview of sequencing outcome from four strands experiments.**

Reactions with 250 nM each A:a and B:b duplex										
X:Y	Seq_reads total	Seq_reads after filter1	Seq_reads after filter2	Final Seq_reads after filter3	Final seq_reads remained (P1+P2+P3+P4)	P1	P2	P3	P4	$\frac{(P2+P3)}{(P1+P2+P3+P4)}$
C:G	986419	742264	494097	494043	50 %	102665	147191	62553	181634	42 %
G:C	830715	619338	424258	424220	51 %	106620	119612	86252	111736	49 %
A:U	1117599	825415	593867	593808	53 %	258097	142977	91043	101691	39 %
U:A	1164835	883594	589907	589844	51 %	200243	180783	77364	131454	44 %
G:U	1112853	846177	575240	575178	52 %	162424	129437	132519	150798	46 %
Average					51 %					44 %
Reactions with 5 nM each A:a and B:b duplex										
X:Y	Seq_reads total	Seq_reads after filter1	Seq_reads after filter2	Final Seq_reads after filter3	Final seq_reads remained (P1+P2+P3+P4)	P1	P2	P3	P4	$\frac{(P2+P3)}{(P1+P2+P3+P4)}$
C:G	626598	459541	322580	322545	50 %	82803	5242	1614	284138	2 %
G:C	812403	605961	444034	443997	51 %	122927	8005	2225	189388	3 %
A:U	925752	705117	492870	492821	55 %	220504	7200	2401	213892	2 %
U:A	797618	598905	427343	427299	53 %	256511	9914	3641	222755	3 %
G:U	721144	531213	370581	370556	54 %	190328	12147	12158	212666	6 %
U:G	626598	459541	322580	322545	51 %	95968	7713	1491	265384	2 %
Average					52 %					3 %

**Table S4. Global overview of sequencing outcome from two-strands reactions.**

Reactions with 5 nM A:a duplex concentration (replicate 1)					
X:Y	Seq_reads total	Seq_reads after filter1	Seq_reads after filter2	Final Seq_reads after filter3	Final seq_reads remained, P1
C:G	1557292	1047742	665331	665093	43 %
G:C	1320317	873066	506164	505983	38 %
A:U	1905215	1258230	923163	922777	48 %
U:A	1817511	1252735	811183	810877	45 %
G:U	1722321	1177677	765060	764788	44 %
Average					44 %
Reactions with 5 nM A:a duplex concentration (replicate 2)					
X:Y	Seq_reads total	Seq_reads after filter1	Seq_reads after filter2	Final Seq_reads after filter3	Final seq_reads remained, P1
C:G	474423	348181	217834	217818	46 %
G:C	1136061	793258	502059	501912	44 %
A:U	1312952	956260	695205	695008	53 %
U:A	1225927	929016	586413	586279	48 %
G:U	1023161	758386	507323	507238	50 %
U:G	142720	104000	53839	53836	38 %
Average					47 %

**Table S5. Representative sequencing results of loop-closing product P1.** Reads are normalized by  $\alpha_1$  (Table S2), direct readout is available in Supplementary excel file. Sequence patterns are colour-coded for the top 40 and bottom 40 sequences in each column.

Rank order	C:G closing	Reads	G:C closing	Reads	A:U closing	Reads	U:A closing	Reads	G:U closing	Reads	U:G closing	Reads
1	UGCG	5866	UCCG	10167	GAGA	16747	GGGA	13314	CUUG	16966	UUCG	1946
2	UGUG	4779	UACG	10057	GGGA	13633	GAGA	12983	CUCG	16167	UGCG	1599
3	UUCG	4683	UCUG	10006	GUGA	11903	UCCG	12188	CUAG	15888	UUUG	1561
4	UUUG	4516	UCAG	9589	GCGA	11857	UUCG	12147	CGCG	15235	UUAG	1451
5	UACG	4323	UGAG	9375	CUCG	10271	GUGA	11938	CUGG	15071	UCCG	1373
6	UCCG	4261	UGCG	9288	UGCG	9892	UGCG	11661	UUCG	13896	CUGG	1207
7	UCAG	4246	GUGA	9145	UUAG	9627	GCGA	11156	CGAG	11602	CCCG	1141
8	UUAG	4206	GAGA	8940	CCCG	9410	UUUG	10411	GUGA	11580	GAGA	1117
9	UGAG	4027	UUUG	8495	CGCG	9368	UACG	9295	CACG	11097	CGUG	1103
10	UAUG	3594	UUCG	8376	CGAG	9158	UCUG	9099	CGUG	10659	UGUG	1094
11	UUGG	3541	GGGA	8365	CUGG	9143	UUAG	8994	UGCG	10475	UUGG	1076
12	UCGC	3516	UUGG	8189	UACG	9016	CCUG	8670	GCGA	10330	CCUG	1068
13	UAAG	3442	UAUG	8051	UGAG	8950	CACG	8131	CGGG	10288	CGAG	1038
14	UUGC	3405	GCGA	8036	UUUG	8916	UCAG	8082	CCCG	10069	GGUC	1035
15	UCGG	3282	UAAG	7778	CACG	8810	CCCG	7865	GAGA	9934	GUGA	1014
16	CCUG	3187	UGUG	7595	CAAG	8778	UUGG	7730	CCUG	9895	GGGA	1003
17	CAGG	3067	UUAG	7588	CUAG	8715	CGCG	7710	GGGA	9824	GCGA	976
18	UCUG	3065	CCGG	7532	CUUG	8604	UGUG	7677	CAUG	9749	CCAG	971
19	CGUG	2899	CUCG	7455	UCAG	8567	UGAG	7617	CCGG	9409	CUAG	960

20	UAGC	2888	CCUG	7375	CAGG	8468	CAUG	7546	UACG	9226	CACG	937
21	CUCG	2862	UCGG	7325	UUGG	8416	UUUA	7468	CCAG	9219	CUCG	907
22	GGGA	2839	CUUG	6983	UUCG	8346	CUGG	7396	UCCG	8659	CGCG	888
23	CUGG	2801	CCCG	6907	CGGG	8167	CAGG	7389	GUCA	8441	CUUG	876
24	CGGG	2751	CGGG	6885	UUUA	8088	CGUG	7197	CAGG	8343	CCGG	809
25	CAAG	2730	CGCG	6852	UAAG	8047	CCGG	7180	CAAG	8003	UACG	808
26	CGCG	2727	UAGG	6802	UUGA	7965	CUCG	7065	UUAG	7935	CAUG	805
27	UGGG	2721	CACG	6466	CGUG	7892	UCGG	6818	UUCA	7701	UGAG	803
28	CGAG	2709	CUGG	6457	UGUG	7846	CUUG	6779	GUUA	7586	CGGG	802
29	CACG	2556	CGUG	6378	CCAG	7747	CGGG	6777	UGCA	7554	UCUG	787
30	UCAC	2544	UCUA	6296	CCUG	7738	UAUG	6723	GGUA	7525	CAAG	785
31	CAUG	2536	UGGG	6229	GAAA	7514	GAAA	6717	GUUC	7254	UGGG	665
32	GAGA	2502	CUAG	6100	UCCG	7416	CGAG	6469	UUUG	6929	GAAA	605
33	UAGG	2489	CAGG	6019	UCUG	7327	CAAG	6298	GGCA	6796	UAUG	599
34	GUGA	2485	CCAG	5970	CAUG	7186	CCAG	6132	UUGA	6796	UCAG	579
35	CCAG	2434	GAAA	5934	UUCA	7150	UAAG	5775	UCUA	6738	CAGG	576
36	CCCG	2430	UUCU	5911	CCGG	7126	CUAG	5655	GAAA	6584	UCGG	572
37	CCGG	2426	CGAG	5820	UAGG	6953	GUAG	5482	UUUA	6527	GGAA	552
38	UAAC	2402	UUUU	5754	UCGG	6887	UUGC	5464	UGGA	6515	UAGC	514
39	UGGC	2348	CAUG	5742	UGGG	6780	UGGG	5323	GUGG	6453	UAGG	500
40	UUAC	2326	GCUA	5637	UAUG	6500	UUUU	5287	GCUA	6243	UUGC	466
41	CUUG	2300	UUGA	5590	GUCA	6060	UAGG	5217	UCAG	6231	GGUA	457
42	CUAG	2299	GGAA	5438	UUUU	5959	UCGC	5215	CUUC	6174	GUAG	448
43	UGAC	2211	GCAA	5257	UCGA	5623	GCAG	5176	UGUA	6083	UAAG	443

44	GCGA	1952	CAAG	5018	UGGA	5454	GAAG	5037	UCCA	6038	GUCA	399
45	UGCA	1874	UCUU	4883	UCCU	5361	GUCA	4916	GCAA	5848	UUUA	391
46	GUAG	1866	GAUA	4605	UAUA	5355	UAGC	4862	UGAG	5785	UGGC	387
47	UGUA	1765	GGUA	4310	UUCU	5257	UUGA	4759	UCGA	5778	GUAA	365
48	UCGA	1577	UCGC	4249	UUAA	5133	UAAC	4667	UUGG	5777	GCAA	364
49	UACA	1458	UUGC	4234	UUGC	4941	UUCU	4485	CUAC	5759	UUUU	356
50	UUGA	1373	UUAU	4197	UAAC	4940	UUCA	4346	UGUG	5678	UAAC	347
51	CUGA	1329	UGUU	4179	GUUA	4817	UCUA	4269	UCUG	5514	UCGC	342
52	UGUC	1327	UUGU	4137	UCGC	4776	GGUA	4234	CGGA	5492	GGAG	332
53	UUCU	1326	GUUA	4105	GGCA	4693	UGAC	4202	UAGG	5489	GCAG	324
54	GGUA	1293	UAUA	4052	UGAC	4652	GUUA	4181	GGAA	5424	UGUA	321
55	UCUA	1292	UUCA	4019	UUAC	4651	GGCA	4016	GUAC	5289	GAAG	317
56	UUUU	1292	UGUA	3971	UGCA	4558	GGAG	3960	CGCA	5229	GUUA	309
57	CGGA	1244	UGCU	3941	UGUA	4549	UUAC	3819	GUCC	5222	UUAC	304
58	UAUA	1238	UCAC	3914	UAGA	4543	UGUA	3765	UACA	5116	UUCA	301
59	CAGA	1203	UAGC	3867	GGAA	4536	UGGC	3705	CUCA	5012	UGAC	294
60	UUUC	1187	UAUU	3861	UUAU	4429	UGGA	3548	GUAA	4946	GGCA	291
61	UGCU	1179	UGAU	3841	UCUA	4425	UGCA	3443	UCGG	4931	UUAU	278
62	UGCC	1175	UAAC	3823	UCAC	4327	UCCA	3428	UUAC	4868	GUUC	277
63	UUCC	1157	GUCA	3784	UAGC	4312	UCGA	3413	CUGC	4862	GGUG	268
64	GGAA	1154	UCGA	3756	UUGU	4290	GACA	3329	UAUA	4857	UCAC	266
65	UUAU	1148	UUUA	3727	GUAC	4284	UAUA	3234	GCCA	4750	UAUA	260
66	GUUG	1138	UGAC	3631	GUAG	3849	UAGA	3042	CACA	4659	GUGG	254
67	GCAG	1126	UCAU	3581	UCCA	3840	GUUC	3023	GGUC	4484	UGUC	248

68	CAAA	1101	UCGU	3494	GACA	3751	GGAA	2959	CGUA	4348	UGCA	247
69	UUCA	1075	UGGA	3478	UGAU	3751	GAUA	2913	CGUC	4264	UCUA	241
70	UAGA	1061	UGCA	3462	UACA	3718	UCAU	2903	CUCC	4235	UUGU	233
71	CCGA	1040	UUAC	3429	GUUC	3700	GUGG	2853	CUUA	4213	GCUA	233
72	UGUU	1037	GUAA	3354	GUAA	3603	UUGU	2824	UUGC	4134	UUCU	232
73	UGGA	1000	GGCA	3348	UCAU	3596	UUAU	2751	CGAC	4125	GAUA	231
74	UUGU	997	UCCA	3347	GGUA	3465	UGUU	2628	GUGC	4061	UUGA	226
75	UCCA	993	UAGA	3311	UGAA	3462	UGAU	2610	CUGA	4052	GUAC	223
76	CUGC	981	UCCU	3251	UAAA	3450	CUCA	2603	UGGG	4030	UGGA	221
77	CGAC	980	UCAA	3242	UGGC	3446	GCUA	2582	UAAG	3890	GUCG	218
78	GGUG	965	UGGU	3202	GUCC	3371	UACA	2556	CUAA	3831	UUUC	216
79	UAUC	964	UACU	3152	UCGU	3274	UUCC	2549	CCCA	3828	UAAU	211
80	CGAA	951	UACA	2796	UGCU	3089	GAGG	2519	GACA	3747	UUCC	209
81	GAAG	942	UUUC	2724	UGGU	3077	UUUC	2392	UUCC	3742	UGUU	208
82	GUCG	933	GUUC	2620	GCAG	2984	UCGU	2375	UAUG	3737	UGGU	208
83	CUAC	907	UGGC	2589	UUUC	2976	GCCA	2369	UCAA	3711	GUUG	204
84	CUAA	888	UGAA	2557	UUCC	2976	UCUU	2367	GUUG	3690	UGAU	199
85	GUGG	882	UAGU	2458	GCUA	2971	UAAU	2350	UGAC	3680	GGGG	191
86	UUUA	876	UAUC	2428	GCCA	2913	CGCA	2323	GAUA	3663	GCCA	183
87	GAAA	856	GUAG	2360	GCAA	2861	GCGG	2322	UCAC	3596	UCCA	176
88	GGAG	854	GUAC	2359	GAAG	2811	GGGG	2276	UUUC	3596	UAGU	173
89	CGCA	852	UAAU	2322	GGAC	2811	GUCG	2248	GUCG	3561	GGUU	171
90	UCGU	843	UGUC	2230	CUCA	2794	UCCU	2235	GCAG	3529	UAGA	168
91	CUCA	842	UUCC	2201	CGGA	2746	UGGU	2217	UGAA	3507	UCGA	168

92	CGUA	833	GGUC	2076	UCUC	2730	GGUG	2178	GCUC	3489	GCGG	162
93	UGGU	833	GCCA	2074	UACC	2711	GUAA	2176	GAUG	3487	UCGU	154
94	GUCA	822	GUUG	2024	UAAU	2694	GUCC	2171	UCGC	3463	GACA	153
95	UACC	821	CUUC	1980	CUUA	2692	CCCA	2118	GUAG	3401	UCAU	153
96	GGCA	814	UCUC	1975	UAUC	2676	GUAC	2117	CCGA	3394	GAGG	151
97	CCUA	810	UACC	1914	UGCC	2656	UAGU	2055	UCCU	3384	UGCC	150
98	AUAG	801	CUUA	1868	UACU	2638	CUUA	2053	UAGA	3365	CUGA	149
99	UGAU	792	CUCA	1864	UGUC	2611	CACA	2027	CGCC	3304	GGAC	149
100	CAAC	763	UGCC	1861	GGAG	2445	UCAC	2003	CAUC	3297	CUUA	148
101	UCAU	761	AUUU	1836	CUUC	2436	UGCU	1988	UGUC	3294	CUAA	148
102	UCUU	755	CUAC	1820	CUAA	2427	UCCC	1965	UAAC	3256	AGUA	147
103	UCUC	754	UUAA	1775	CUAC	2424	AUUA	1959	AUGA	3210	GGCC	147
104	CCAC	739	GACA	1759	UAGU	2384	AGUA	1956	UGCC	3185	CCGA	141
105	UGAA	713	GUCC	1727	GGUC	2362	GUUG	1932	GCUG	3168	GAUC	140
106	CACA	703	UAAA	1706	UGUU	2301	GGUC	1887	GGGG	3154	GGGC	137
107	UAUU	699	GGUG	1565	CCCA	2257	CUCC	1870	GGCG	3064	GCCG	136
108	CAGC	692	CUAA	1557	CUCC	2148	UCUC	1829	AGUA	3050	UCUU	134
109	CCCA	672	GGAG	1545	GGGG	2145	CGUA	1828	GGUG	3041	CGUA	132
110	CCAA	661	UCCC	1526	CGAC	2142	CGGA	1806	GAUC	2991	CUCA	131
111	UCAA	658	CCUA	1487	UAUU	2092	UGUC	1791	UUGU	2985	GUCC	130
112	UAAU	631	GAUG	1441	CUGA	2089	CUGA	1746	UAGC	2977	GCUG	128
113	CGGC	624	GGAC	1431	AUUA	2038	UAUC	1739	CGGC	2973	UACA	127
114	CCGC	608	GCAG	1419	UCAA	2035	AUCA	1738	GCCG	2950	AUAG	125
115	AUCG	607	CUCC	1367	GUGG	2008	UGCC	1733	GGAG	2934	UAUC	123

116	UACU	606	AUCU	1346	CGCA	1986	UAUU	1703	CCUA	2886	GUAU	120
117	CUUC	605	GUGC	1342	CACA	1960	CCUA	1625	GGAC	2851	GGCG	119
118	CUUA	605	GUCG	1294	UCCC	1922	GUCU	1562	UGGC	2840	AUUA	118
119	GGCG	580	CGUC	1250	CAUC	1881	UAAA	1562	CGAA	2839	GUGC	115
120	CAUA	579	CGAC	1238	AUAA	1866	CUUC	1544	GGCC	2825	GUGU	114
121	UCCC	567	GAAG	1226	CGUC	1843	UACC	1509	GCGG	2823	CUUC	113
122	GUAA	563	CGCA	1208	CGUA	1812	CAUA	1495	CAAC	2798	UUAA	111
123	GCAA	550	AUUA	1205	CAAC	1804	AUGA	1484	UCUC	2786	CGCA	110
124	GACA	542	GUGG	1203	GUGC	1769	CUAC	1480	GUGU	2763	CAGA	109
125	CUCC	533	CAUC	1174	AUCA	1738	CAGA	1476	UCCC	2714	UAUU	105
126	CGUC	522	CCCA	1173	AGGA	1737	UGAA	1476	UGCU	2706	UCUC	104
127	UCCU	522	CCAC	1173	CAAA	1707	CCCC	1427	UCGU	2665	CGGA	102
128	UAAA	506	CCGA	1164	CUGC	1672	AUAG	1398	CCUC	2617	CGUC	102
129	UAGU	505	CUGA	1160	AGUA	1669	AUUU	1384	UACC	2563	GAUG	101
130	GUUA	500	CCAA	1154	GUGU	1668	CUAA	1357	CACC	2555	AGUC	100
131	GUAC	494	AUAU	1149	GAGG	1656	CCGA	1345	UUAA	2525	AUCA	97
132	GGGG	478	CUGC	1121	CCAC	1644	CAAA	1341	CCGC	2521	GCAU	97
133	CUGU	470	AUAA	1108	CACC	1609	GCAU	1326	CUCU	2489	CAUA	97
134	GAUA	460	AUCA	1108	CCGA	1596	GCUG	1296	CCCC	2462	CCCA	95
135	CGCC	450	CCUC	1093	CCUA	1589	GGCG	1283	CCAC	2458	AUGA	95
136	GUUC	436	CUGU	1070	GGCC	1587	UACU	1280	GAAG	2455	GGAU	94
137	GCUG	435	AGUU	1069	CUGU	1553	GGAC	1271	UAUC	2453	AUAA	90
138	UUAA	430	GUAU	1061	CCCC	1521	GUGC	1261	UAAA	2451	CCAA	88
139	GAUG	424	CGGA	1046	GAAC	1507	AUAU	1257	GCCU	2441	AGGA	88

140	GCGG	422	GGUU	1038	AUAU	1479	CGAC	1250	GCGC	2435	UCCC	87
141	CAUC	416	CGUA	1023	GAUC	1476	GCAA	1246	GCAC	2434	CGAA	86
142	GAGG	405	CAUA	1017	GAUA	1476	GUAU	1208	UUCU	2414	GCAC	85
143	GUCC	403	CCGC	1014	CAGA	1447	CAUC	1207	GGGC	2404	UGCU	83
144	CCUC	370	CACA	1012	GUCG	1444	UUAA	1205	CCCU	2394	GGGU	81
145	GGAC	368	GUGU	992	AUGA	1439	AAUA	1199	GCCC	2332	UGAA	81
146	GACG	367	GCUC	991	AUUU	1424	AGGA	1197	AGGA	2327	CUCC	80
147	GCUA	349	GAUC	983	CGCC	1405	GAUG	1192	UGGU	2322	UCAA	77
148	CUAU	347	GUCU	967	GCAC	1384	AGCA	1191	CGCU	2310	CUAC	75
149	GGUC	345	AUAG	965	GCGG	1340	CACC	1159	CAAA	2294	UCCU	72
150	GUGC	344	AGUA	960	CCUC	1312	GACG	1150	UUUU	2276	GCUC	71
151	CACC	343	AUAC	949	GCGC	1285	AUCU	1132	CUGU	2241	GACG	70
152	AGUA	337	CGCC	938	ACUA	1253	CGUC	1124	CAGC	2229	CACA	69
153	CCCC	331	CACC	922	GUUG	1251	CUGU	1096	GCGU	2222	GUUU	69
154	AUGA	325	GGCC	908	UCUU	1243	GGCC	1086	CAGA	2149	CCUA	68
155	AUUG	323	GCAC	896	CAUA	1238	CCUC	1082	CGGU	2149	GUCU	67
156	AUAC	317	GGAU	890	CGGC	1225	GUGU	1051	AUCA	2102	CAAA	67
157	CGGU	306	AUGA	887	AGCA	1194	ACUA	1030	GGCU	2080	AGCA	67
158	AUCA	300	GCUG	884	CGAA	1174	CGCC	1029	GAGG	2079	UACC	66
159	GUAU	298	GGCG	850	AUAG	1145	GUUU	1021	AGCA	2074	AAUA	66
160	AGGA	287	CGGC	849	GACC	1135	ACGA	1020	GUCU	2051	AUCG	66
161	AUCU	284	CAAC	840	CAGC	1128	CAAC	1012	UCAU	2013	CUGC	64
162	AGAG	268	GUUU	840	AUCU	1127	GAAU	1006	CCGU	2006	CUUU	63
163	AUAU	261	CCCC	820	GGCG	1099	GAUC	991	AUUA	1945	CUGU	59

164	GUGU	253	ACUU	812	CCGC	1084	CUGC	977	AUUU	1945	AUAU	59
165	ACAG	246	CGAA	808	GAUG	1080	GCUC	962	ACUA	1917	AGAA	58
166	CUCU	244	GGGG	802	GGGC	1067	CAGC	937	UGAU	1866	GCGU	58
167	CGAU	242	GAGG	793	ACGA	1054	AUGU	912	GACC	1842	AUUG	55
168	CGUU	242	CAGA	788	AUAC	1043	CUCU	901	GACG	1824	AUGG	54
169	GCCA	241	CGGU	777	CGGU	1019	AUCG	899	AUUC	1820	CCGU	53
170	AGCA	239	CUUU	777	GUCU	965	AGUU	891	ACGA	1779	UAAA	53
171	AUUA	239	GCAU	767	GGAU	953	UCAA	879	AUAU	1768	GCGC	52
172	GGUU	239	CAGC	767	AAAA	944	CGGC	859	UAGU	1746	CUCU	52
173	GCCG	236	CUCU	763	GUAU	933	CGAA	847	GGGU	1732	CCUC	51
174	AUUU	232	AGAA	762	GCCC	914	CUUU	839	UACU	1713	CGAC	50
175	CCCU	231	CCGU	749	GACG	912	GGGC	835	AUCU	1703	AGUG	50
176	AUGU	214	GCUU	747	CCCU	908	ACCA	831	AUAG	1702	GAAU	50
177	AUGG	213	CCUU	738	ACAA	894	GCCG	818	ACAA	1673	CGGC	50
178	ACGA	210	GGGU	729	CUCU	890	GCCC	803	AUGG	1659	AUUU	50
179	CUUU	208	GCGU	726	CUUU	886	AUAC	800	AUCG	1659	CCCC	49
180	CCAU	207	GGGC	719	AGAA	884	GAAC	782	UGUU	1652	AUCU	48
181	GGGC	206	AUGU	715	AAUA	877	GCGU	774	AUGC	1644	GAAC	48
182	CCUU	206	GCGG	715	ACCA	870	CCGC	764	GCAU	1620	CCGC	46
183	AUUC	204	AGGA	700	GCAU	857	GCGC	751	CAUA	1607	AGUU	46
184	ACUA	204	CUAU	696	GCGU	853	GGAU	751	ACCA	1604	AUGU	46
185	GUCU	204	GAAC	695	CCGU	829	GCCU	747	AUAC	1587	AAGA	45
186	CCGU	199	GAAU	690	GGUG	817	CGGU	739	AUUG	1573	CUAU	45
187	GUUU	194	ACGA	682	GCUG	817	CCCU	737	GAGC	1477	CGCC	45

188	CGCU	194	AGCA	680	GCUC	816	GGGU	732	GAAC	1475	CGGU	44
189	CAGU	190	CAAA	676	AUGU	806	AUUG	730	AGUC	1468	GAGC	44
190	CACU	189	GAGU	663	GGGU	800	CCGU	718	GGUU	1455	GCCU	44
191	GGGU	187	ACUA	660	ACCU	769	GACC	714	ACUG	1424	GAGU	43
192	AGAA	184	GAUU	655	GAAU	764	CUAU	687	CACU	1418	GAUU	43
193	GGCC	184	AUUG	648	AUCG	755	CCUU	681	CUAU	1400	GACC	42
194	AGAC	183	AAUA	635	CUAU	755	ACUU	680	GUAU	1392	ACCA	42
195	AGCG	182	GCCC	600	GUUU	725	GGUU	674	AUCC	1372	ACUA	42
196	AGUU	182	ACAA	593	GAGC	715	CGUU	673	UAAU	1370	CCAC	41
197	AUGC	179	GGCU	588	AACA	702	AGAA	665	CUUU	1356	CCUU	40
198	AUCC	176	CAUU	581	GCCU	688	AUAA	662	GGAU	1347	GGCU	39
199	GGAU	174	AUCG	574	GAGU	688	GAGU	649	ACCU	1328	CACC	39
200	AGUG	173	CAGU	573	GGCU	634	AAGA	634	ACCG	1320	AUGC	38
201	CAAU	172	GACC	550	ACAU	626	CAGU	624	ACGG	1302	GCCC	38
202	ACUU	167	GCGC	547	AUUG	590	CCAU	616	CCAU	1250	AUAC	37
203	ACUG	166	AAUU	526	AUUC	589	CGAU	611	AGUG	1241	CAUC	37
204	CAUU	163	CCCU	494	AAGA	589	CGCU	610	AGCG	1238	AGAG	36
205	GCGC	160	ACAU	484	CCAA	584	AUUC	600	CGUU	1218	ACAA	35
206	AAUA	157	CGUU	476	AGAU	578	CACU	587	ACUC	1210	AUUC	34
207	AUAA	156	AGGU	476	GCCG	567	AACA	580	GUUU	1206	AAUU	33
208	GCUC	154	CCAU	473	CAGU	553	AAAA	574	GCUU	1168	ACAG	33
209	ACAC	153	GCCU	472	ACAC	527	AGUG	570	ACAG	1165	CAUU	33
210	AGAU	153	ACCU	471	AGAG	526	AUCC	569	CCAA	1160	AGGG	33
211	GCAC	151	CACU	470	AGGU	520	AUGC	559	UUAU	1152	UACU	33

212	AACA	147	AGAU	467	AUGC	512	CCAA	542	ACGC	1150	ACGA	33
213	ACCA	147	AUGC	465	AUCC	505	AUGG	537	AUGU	1120	AAAA	32
214	AGUC	140	GAGC	441	CACU	503	GAGC	525	ACCC	1108	CGUU	32
215	GAAC	139	AUUC	432	AGCU	501	GGCU	523	UCUU	1091	CAGC	31
216	GCAU	139	GCCG	429	CGAU	487	CAUU	507	GAGU	1086	GACU	31
217	GCGU	139	ACCA	408	ACGU	482	ACAG	497	CGAU	1082	ACCG	30
218	AGGU	138	AGCU	403	CCAU	473	ACAU	486	CCUU	1021	GCUU	30
219	GAUC	136	AGUC	388	CGCU	471	CCAC	485	AGGG	996	AAUG	30
220	AACG	133	AGAG	384	ACAG	464	ACGU	481	AAUA	985	CAAC	29
221	ACAU	127	CGCU	375	AGUU	446	AAUU	469	AGAG	982	CCCU	28
222	AAGA	125	ACAC	374	AGAC	444	AGGU	463	AGAA	977	AGAU	27
223	GAGU	123	GACG	366	CAAU	438	AGAG	463	AAAA	974	AGGU	27
224	AGCU	122	ACAG	351	GGUU	432	ACCC	455	UAUU	974	ACUG	26
225	ACCG	121	AGAC	340	AGUC	428	AGAU	438	ACGU	973	AAGG	26
226	GGCU	120	ACGU	335	GACU	411	AGCG	434	AGGC	969	CGAU	25
227	AAUG	119	AUCC	331	AUGG	410	ACCU	432	AGCC	962	AGCU	24
228	GCUU	118	AAAA	326	ACUU	386	ACCG	421	AGGU	949	CAGU	24
229	GAAU	111	GACU	324	AGCG	375	CAAU	420	AGCU	941	AUCC	24
230	AAAG	104	CGAU	306	ACGC	363	AGCU	416	GACU	934	ACUU	23
231	GACC	104	AACA	300	CGUU	350	GCUU	407	ACAU	918	ACAU	23
232	ACGC	103	AAGA	300	ACUG	340	GAUU	398	AACA	909	ACAC	22
233	AGGG	103	AUGG	292	CAUU	336	ACUG	387	ACAC	886	AGCG	22
234	ACAA	103	AGUG	275	ACGG	309	GACU	386	CAGU	886	CCAU	22
235	ACGU	100	ACGC	249	AGGC	301	ACGC	377	ACUU	808	CACU	21

236	GAGC	98	CAAU	248	AGGG	291	AGUC	351	AGUU	781	AACA	21
237	GAUU	97	AAUG	247	AGUG	285	ACAC	333	AGAC	752	CAAU	21
238	AGGC	92	AGCG	245	AACU	284	GCAC	333	AUAA	748	ACCC	18
239	ACUC	92	ACUG	233	AAAU	280	ACAA	322	AAUG	745	ACGG	18
240	GCCC	85	AGGC	231	GAUU	276	AGAC	322	AGAU	744	ACGU	17
241	AAUU	83	AACU	223	ACCG	268	AGGG	317	AAGA	709	AAAG	17
242	AACU	81	ACCG	218	GCUU	258	AAUG	316	GAAU	704	AGCC	17
243	AAAA	80	AGGG	206	AGCC	257	ACUC	302	AAUC	623	AGAC	17
244	ACGG	77	ACUC	201	ACUC	256	AACU	301	GAUU	603	AAUC	16
245	GACU	76	AGCC	199	AAUC	252	ACGG	299	CAUU	577	AGGC	14
246	AGCC	74	AAGU	194	ACCC	250	AGCC	290	AACG	551	AAGC	13
247	GCCU	72	AAUC	175	AACG	246	AGGC	286	AAAG	475	ACCU	13
248	ACCU	70	AAAU	170	AAUU	245	AACG	264	CAAU	474	AAAU	12
249	AAAC	65	ACGG	167	AAGU	230	AACC	238	AACC	438	CGCU	12
250	ACCC	65	ACCC	162	AAAC	222	AAAU	234	AAGG	407	ACGC	11
251	AAUC	63	AAAC	142	AAUG	219	AAAG	232	AAAU	386	AAGU	11
252	AAAU	55	AAGC	129	AAAG	195	AAUC	215	AACU	366	ACUC	11
253	AAGC	49	AAAG	128	CCUU	181	AAAC	210	AAGC	363	AACG	11
254	AAGU	49	AACG	124	AAGC	164	AAGU	188	AAUU	339	AACU	11
255	AAGG	46	AACC	113	AACC	163	AAGC	169	AAGU	329	AACC	8
256	AACC	45	AAGG	92	AAGG	139	AAGG	156	AAAC	303	AAAC	7
Frequency Range		130		111		120		85		56		278

**Table S6. Comparison of the top 40 sequences of loop-closing product P1 from reactions at 250 nM and 5 nM duplex A:a concentration.**

Newly appeared sequences in the 250 nM reactions comparing to the 5 nM ones are highlighted in red.

Rank	X:Y = C:G		X:Y = G:C		X:Y = A:U		X:Y = U:A	
	Reaction concentration at		Reaction concentration at		Reaction concentration at		Reaction concentration at	
	250 nM	5 nM	250 nM	5 nM	250 nM	5 nM	250 nM	5 nM
1	UCAG	UGCG	UUGG	UCUG	GAGA	GAGA	GAGA	GGGA
2	UUAG	UACG	UCAG	UCCG	GUGA	GGGA	UACG	GAGA
3	UUGG	UGUG	GUGA	GAGA	GGGA	GUGA	GGGA	GUGA
4	UGAG	UUCG	UACG	UCAG	GCGA	GCGA	UUCG	UCCG
5	UGCG	UUUG	UGAG	UACG	UUGG	CUCG	GUGA	UUCG
6	UACG	UGAG	GUGG	GUGA	CUCG	UUAG	GCGA	UGCG
7	UUUG	UAUG	GAGA	UGAG	UUAG	UGCG	UUUG	GCGA
8	UCUG	UUAG	UUUG	UGCG	CGCG	UGAG	UCCG	UUUG
9	UUCG	UAAG	UUCG	GGGA	UGAG	CUGG	UUAG	UCUG
10	UUGC	UCAG	UCUG	UAUG	CACG	CGCG	UGCG	UUAG
11	UAUG	UCCG	UAAG	UAAG	UGCG	CUAG	UCAG	CCUG
12	UAAG	UUGG	UUAG	UGUG	CUAG	UACG	UCUG	UUGG
13	UCGG	CAGG	GCGA	UUAG	CCCC	CGAG	UGAG	UGAG
14	UGUG	UAGC	CUCC	UUCG	CCC	UUUA	UUGG	UGUG
15	UCGC	UUGC	CUGC	UUGG	UCGG	CCC	CACG	CAUG
16	UGGG	CGUG	UAGG	GCGA	UAGG	UUUG	CUCG	UCAG
17	UAGG	CUGG	UAUG	UAGG	CUGG	CUUG	UCGG	UACG
18	UCAC	CCUG	UCCG	UCGG	CGAG	UUCG	UAUG	UUUA
19	UCCG	GGGA	CCCU	CCGG	CUCC	CAGG	CGCG	CACG

20	UAGC	UCGC	CUCG	UCUA	UCAG	CAAG	UGUG	CAGG
21	CUGG	UAAC	UGCG	CCUG	UAAG	UUGG	CCUG	CUGG
22	UGAC	CGAG	CUGG	UUUG	UGGG	CACG	UAAG	CGUG
23	CGGG	CAAG	UGUG	CGGG	UUCG	UAAG	UGGG	CCCG
24	CCGG	UGGC	CCUC	CUCG	CUUG	UGUG	CCGG	CCGG
25	UGGC	CAUG	CCUG	GAAA	UACG	UCAG	UCGC	CGGG
26	UAAC	UAGG	UCGG	UUCU	CAGG	CGGG	CUGG	UCGG
27	GGGA	CGGG	GCUA	CUGG	UUUG	UUGA	CAGG	CUUG
28	CCCG	CACG	CACG	CCCG	CGGG	GAAA	UUGC	GAAA
29	CAGG	CGCG	GGGA	UUGA	CAAG	CCAG	CAUG	UAUG
30	GCGG	UCGG	UGGG	CACG	CCCA	CGUG	GAAA	CGCG
31	GCGG	UCUG	CGCG	CCAG	UUGA	UUCA	GCGG	CAAG
32	GCCG	CUCG	CUAG	GGAA	UCCG	UCCG	GAAG	CCAG
33	GGGG	CUAG	CUUG	UCUU	CCGG	CAUG	UAGG	CGAG
34	UUAC	CCCG	GCUG	UGGG	CCAG	CCUG	GUAG	CUCG
35	CGCG	GUGA	CGGG	CUUG	GCCC	UAGG	CUUG	UAAG
36	CGUG	CCAG	GUCG	UUUU	GUCA	UGGG	CGAG	GUAG
37	CGAG	UGGG	UCCC	CAGG	GAAA	CCGG	CCCG	UUUU
38	CCUG	UGAC	CGAG	CUAG	UUUA	UCUG	CCCU	UGGG
39	UGCC	UUAC	CCGG	CGUG	UUCA	UCGG	CUAG	UUGC
40	CCGA	CCGG	UUGC	GCUA	GCGG	UAUG	CAAG	UCGC

**Table S7. Top 40 and bottom 40 sequences of splint ligation product P2 from reactions at 250 nM duplex A:a concentration.** Sequences having less than 3 Cs or Gs among the top 40, and having less than 3 As or Us among the bottom 40s, are highlighted.

Rank order	X:Y = C:G	Reads	X:Y = G:C	Reads	X:Y = A:U	Reads	X:Y = U:A	Reads	X:Y = G:U	Reads
1	CCCC	4501	CCCC	3643	CCCC	4246	CCCC	5182	GCCG	4007
2	GCGG	4371	GCCC	3305	GCCC	4226	GCGG	4993	GCGG	3841
3	CUCC	4153	GCGG	3011	GCGG	4214	GCCC	4900	GCCC	3517
4	GCCC	4075	CCGC	2960	GGGG	3892	GGGG	4453	GCCA	3385
5	UGCC	4039	CCCA	2945	GGCG	3761	GCCG	4313	GGGG	3285
6	GCCG	3834	CGCC	2904	GCCG	3569	GGCG	4253	CCGG	3220
7	CCGC	3796	GGGG	2803	CUCC	3343	CUCC	4152	GGCG	3115
8	GGCG	3786	CCGG	2784	UGGG	3228	CCGC	4081	CCCC	2969
9	CCCA	3588	CUCC	2687	CCGC	3216	GGCC	4077	GCGC	2949
10	CCCU	3518	GCGC	2638	CCCA	3110	CCGG	4045	GCAG	2924
11	UCCC	3502	GCCG	2610	CCGG	3030	CCCA	3998	CCCA	2801
12	UGGG	3489	GGCC	2515	CGCC	2965	CGCC	3810	CCCG	2718
13	GGGG	3476	CCCG	2477	GGCC	2876	CCUC	3523	GGCC	2648
14	CGCC	3384	GGCG	2475	CCCG	2874	UGGG	3482	GUGG	2624
15	CCGG	3334	UGCC	2419	GUGG	2842	GCGC	3479	UCGG	2616
16	CCUC	3296	CCGA	2414	UGCC	2815	CCCG	3251	UGCG	2488
17	CUGC	3144	UGGG	2379	GCGC	2792	GCCA	3236	GGCA	2438
18	UGGC	2943	CGGC	2377	CCUC	2693	UCGG	3221	GCUG	2387

19	CCGU	2927	CUGC	2319	CCCU	2596	CUGC	3180	UCCG	2377
20	UCGG	2742	CCUC	2256	CCGA	2436	UGCC	3139	GGGC	2342
21	CCGA	2697	UCCC	2227	GCCA	2429	CCGA	3091	UGGG	2281
22	GGCC	2626	UGGC	2162	UCCC	2425	CCCU	3084	CGCC	2245
23	UCCG	2606	UCGG	2157	UCGG	2404	GGGC	2884	GGGA	2230
24	CCCG	2519	GGGC	2143	UCCG	2313	GUGG	2840	CCGC	2195
25	GUGG	2404	CACC	2102	CCGU	2258	CCGU	2812	GCGA	2169
26	GCGC	2397	GCCA	2016	CUGC	2245	GCAG	2756	GAGG	2123
27	UGCG	2384	GUGG	1966	CGGC	2228	UCCC	2713	CCGA	2121
28	CGGC	2303	CCGU	1963	GGGC	2191	UGCG	2671	GGAG	2066
29	CGCU	2237	CGGU	1926	UGGC	2134	UCCG	2606	ACCG	1910
30	CGGU	2139	GCAG	1886	UGCG	2014	GGCA	2538	CGGG	1874
31	CACC	2115	CCCU	1756	GCUG	2007	GCUG	2444	GGUG	1811
32	CGGG	2109	CCAC	1701	GCAG	1876	ACGG	2414	GCAC	1797
33	GCCA	2092	UCCG	1593	CACC	1861	CGGC	2414	ACGG	1771
34	CCAC	1957	UGCG	1560	CCAC	1820	GUCG	2341	CUCC	1707
35	CGUC	1917	CGUC	1547	CGGU	1721	UGGC	2334	GUCG	1699
36	CCUA	1846	CGGG	1532	GGCA	1714	ACCG	2330	CCCU	1695
37	GGGC	1662	GGCA	1527	CGCU	1714	CACC	2328	UGCC	1671
38	GCAG	1580	CGCU	1476	GUCG	1649	CCAC	2314	GACG	1620
39	GCUG	1566	GAGG	1458	CCUA	1638	CCUA	2288	UCCC	1528
40	GGCA	1558	CGGA	1435	CGGG	1586	GGUG	2176	CCUG	1498
217	GUAA	8	CAAA	15	UAAC	11	CAAU	17	UAAC	13
218	GAAC	8	CUAG	15	CAAA	10	UUCU	16	ACAA	12

219	UUUC	8	CAAG	13	AAGC	9	CAAA	15	AAUG	11
220	GUUA	8	ACAU	12	UUUC	9	AUCU	15	CAAU	10
221	ACAA	6	AUGU	11	AAUG	9	UUUC	14	AACA	9
222	AAUG	6	UAUA	9	ACAA	9	UAAC	13	GAAA	9
223	UAUA	6	ACAA	9	ACUU	8	UGAA	11	AUGU	8
224	CAAA	5	AUUC	9	UAUA	8	AACA	11	CUUU	8
225	AACA	5	AACA	9	ACAU	7	AAGA	10	GAAU	8
226	AAAG	5	CUUU	8	GUUU	7	AUUC	10	AUUC	8
227	UUAU	5	GAUU	7	AACU	7	AUUA	9	CAUU	8
228	AUGU	4	AGAU	7	AAAG	6	AUGU	9	AUCU	7
229	AAAC	3	AGAA	6	AUGU	6	GAUU	7	UUUC	7
230	ACAU	3	ACUU	6	UUAU	6	UUUU	7	ACUU	6
231	ACUU	3	UUAU	5	UAAU	5	ACUU	7	AAGA	6
232	AUUC	3	AAGA	5	GAAA	5	AGUU	6	AGUU	6
233	AGAU	3	GAAA	4	AGUU	5	AGAA	6	CAAA	6
234	UAAU	3	AGUU	4	AUUC	5	UAUA	6	UAUA	5
235	UAUU	3	AAAG	4	AAGA	4	AAUC	6	AAGU	5
236	AAAU	2	AAGU	3	AUCU	4	AAAG	4	AGAA	5
237	AAUC	2	AUUA	3	AGAA	3	AGAU	4	AGAU	4
238	GUUU	2	AAUC	3	AAUC	3	UAAU	4	AAUA	4
239	AAGU	2	GUUU	2	GAUU	3	UUUA	3	AAAG	4
240	UUUA	2	AUCU	2	UAUU	3	AACU	3	UUUA	4
241	AUAU	2	UUAA	2	AGAU	3	GUUU	3	AAUC	3
242	GAAA	2	AAAC	2	AUAU	3	UAUU	3	UUAU	2

243	AGAA	2	UAAU	2	GAAU	2	AUAA	3	UAAU	2
244	AUAA	1	AUAU	1	AAGU	2	AAGU	2	UUAA	2
245	AAAA	1	GAAU	1	UAAA	2	AAAU	2	AAAC	1
246	AGUU	1	UAUU	1	UUAA	2	GAAA	2	AUAU	1
247	UUAA	1	AAUA	1	UUUU	2	UUAU	2	AUAA	1
248	GAUU	1	AACU	1	AUAA	1	GAAU	2	UAUU	1
249	AAUU	1	UUUA	1	AAAC	1	AAAC	2	AACU	1
250	AAUA	1	AAUU	1	UUUA	1	AAUA	1	AAUU	1
251	AACU	1	AUAA	0	AAAA	1	UUAA	1	AUUA	1
252	UAAA	1	UAAA	0	AUUU	1	AUUU	1	UAAA	0
253			AAAU	0	AUUA	1	AAAA	1	AAAA	0
254					AAUU	1				
255					AAUA	1				
256					AAAU	1				
Uncovered Sequence #		4		3		0		3		3

**Table S8. Biological tetraloops from bpRNA-1m(90) tabulated by different closing base-pairs, X:Y.** RNA sequences in the database with > 90% sequences similarities were removed when keeping at least 70% alignment coverage.

Rank order	C:G closing	Counts	G:C closing	Counts	A:U closing	Counts	U:A closing	Counts	G:U closing	Counts	U:G closing	Counts
1	GAAA	1855	GAAA	1433	GAAA	457	GAAA	233	UAGG	31	GAGA	823
2	GCAA	1753	GCAA	640	GAGA	429	GUGA	143	GGAA	24	GAAA	303
3	UUCG	1670	GUAA	481	GCAA	309	GCAA	95	GAAA	23	UUCG	285
4	GUGA	1627	GUGA	480	GUGA	253	UAAC	85	UACG	13	UUAG	112
5	AGCC	867	CUUG	309	GUAA	217	GGAA	75	AAAU	11	UAAC	94
6	UACG	855	GCGA	284	UUUA	194	GUAA	71	UCAU	9	UAGG	83
7	GAAG	771	GGAA	199	AACA	189	UUCG	57	UUAA	8	GGAA	74
8	GCGA	611	GAGA	190	GCGA	165	GCGA	55	UGAU	8	GUGA	66
9	GGAA	453	UGAA	171	AGCA	98	GAGA	48	UUUA	7	GCAA	66
10	UAAC	443	UUCG	96	UCAU	65	GAAG	45	AAAG	7	CAGG	54
11	GUAA	391	ACGA	85	GAGG	44	GCCA	42	UGAG	7	GUAA	53
12	GAGA	376	GCAU	82	GGAA	41	UUUU	39	UAAU	6	GCAU	38
13	CCCC	244	GAAG	67	CUUG	37	AAAA	36	UAAA	6	GGGA	37
14	GCAU	237	GGGA	56	UUUU	30	GAUU	35	AAAC	6	GCGA	34
15	UUUU	191	AGAA	55	UUCG	25	AACA	34	GAGU	6	GCAG	33
16	UUAG	161	UUUA	54	UUAA	23	UUUC	33	AGUU	6	UCCU	30
17	CAAG	159	UUUU	40	AAAA	20	UUAU	30	UAUU	5	UGAG	26
18	GCCA	143	CUUC	40	UUAU	20	GCAG	28	UGAA	5	UAGA	21

19	AGCA	136	ACGG	26	AGAA	16	UUUA	26	UAGU	5	UUUG	21
20	UCCG	130	AAAA	25	UAAA	15	UAAU	22	CCUU	5	ACCU	21
21	UUUG	120	CAUG	24	ACCA	15	UAUA	22	CAAU	5	UCCG	20
22	CUCG	92	UAUA	24	AUAA	13	AUAA	22	GUAA	4	UAAU	17
23	UAAG	91	ACUU	24	AUAU	13	UAAA	21	GCGA	4	CGAA	17
24	AAAC	69	AUGA	19	UCCG	13	GAUA	21	AAUA	4	CUCA	17
25	GGGA	67	ACCU	19	AAAC	12	AAAU	20	UUCA	4	UUUU	16
26	CACG	67	AAGU	18	UAUU	12	AUUU	20	CUUG	4	GAAG	16
27	CUCA	64	CAAG	17	AUUU	12	GACA	19	UGCG	4	ACAG	16
28	CGCC	64	GUCA	17	UUGA	12	AACU	17	AUCA	4	CGCA	16
29	UUAC	57	AUAC	17	AUCU	12	UUAA	16	AAGU	4	UUAA	15
30	GCUA	52	UAAC	16	UAAU	11	AAAG	16	UGUG	4	GAUA	15
31	AACA	50	AAAC	16	UAAC	11	UAUU	15	GUGA	3	AAAA	15
32	AACC	49	UAAA	16	GGGA	11	AAUA	15	GCAA	3	AUCC	14
33	AUUU	48	AGUA	16	GAAG	11	CUUU	15	UUUU	3	UCAC	13
34	UCAC	48	AUUU	15	CUGG	11	AACC	15	GAUA	3	UCAG	13
35	GACA	47	GUUU	15	AAUU	11	UUAG	14	AUUU	3	CUCG	13
36	AAAA	45	AAUU	15	UUGG	10	GUCA	14	UCUU	3	UUAC	12
37	GUCA	44	GUUA	15	UUGU	10	GAAU	14	UGUU	3	CAGU	12
38	UUAU	42	AGCA	14	ACGU	10	CAAA	13	ACAA	3	UAAA	11
39	UAAA	41	AAUC	14	UACG	9	UUUG	12	UCUC	3	ACUU	11
40	CUAG	41	GUGG	14	UCGG	9	AUAU	12	UGCU	3	UCAU	10
41	ACCC	40	UCCG	13	UAGG	8	UUCU	12	UAGA	3	CUAG	10
42	AAUA	39	UUCU	13	UUCA	8	CAUC	12	UUGG	3	CUAA	10

43	GUUU	39	ACAU	13	AGUA	8	AAAC	11	AAGC	3	UACG	9
44	AAUU	38	GGAG	13	CCUC	8	GAGG	11	UAAC	2	UUUA	9
45	UUCU	35	AUAA	13	GGAG	8	AAUU	11	UAUA	2	CUUG	9
46	ACAU	35	UUGA	13	GUAG	8	GACC	10	AUAA	2	AUAG	9
47	UGCG	35	CCUG	13	GUAU	8	UCAU	9	CUUU	2	GGGU	9
48	CACC	35	CUCG	12	GGUU	8	UUCA	9	AACC	2	UUCC	9
49	UGGU	34	GUCU	12	UAGU	7	UCUU	9	UUUG	2	UCCC	9
50	CUUG	33	CGUG	12	AAUA	7	AUUA	9	AUAU	2	UUCA	8
51	UGAC	32	UAAG	11	UCUU	7	GAAC	9	AUUA	2	CAAC	8
52	ACGU	30	CAAC	11	UUUG	7	AAUG	9	GAAC	2	UGCC	8
53	GUUA	30	ACAA	11	AACC	7	UACC	9	AAUG	2	CGUA	8
54	CAAC	30	GAGU	11	UUCU	7	GGAG	9	GGGA	2	CGGG	8
55	UUUC	30	AUUA	9	GGCA	7	UUGU	9	UCAC	2	UUAU	8
56	UUUA	28	GAAU	9	AAAU	6	UGAA	8	CAAC	2	GAAU	8
57	GCAG	28	UAUU	9	UGAU	6	UGUU	8	GUUU	2	UACC	8
58	UUAA	27	AUAU	9	GAGU	6	GGGA	8	GUGG	2	GCCU	8
59	UCAA	27	UUAU	8	UGAA	6	UCAC	8	UUAC	2	GAGU	7
60	GUAG	26	AAAU	8	UUAC	6	CAAC	8	UAAG	2	CCUU	7
61	GGAG	25	CCAA	8	GAAC	6	AGCU	8	AGAA	2	GGAG	7
62	GAUA	25	GUUG	8	GUUA	6	CUCG	8	CCAA	2	CCCA	7
63	UGAG	25	UUAG	7	GUCA	6	UUCC	8	ACUA	2	GAUG	7
64	UUGC	25	UUUG	7	AUCC	6	GUAG	8	GUAC	2	AAAC	6
65	CGGU	25	CGCC	7	AUGA	6	GUAU	8	UGUA	2	AAUA	6
66	GUAU	24	AAUA	7	AGUU	5	UACG	7	UUGA	2	UGUG	6

67	UCAU	23	GCAG	7	GAUA	5	UAGU	7	UGGU	2	UAUA	6
68	UUGU	23	UUAA	7	UAUA	5	CUUG	7	UGCA	2	UGGC	6
69	AAAG	23	UAAU	7	CUUU	5	GUUU	7	ACGA	2	CAAG	6
70	UAUG	22	AACU	7	AUUA	5	GUGG	7	CCAU	2	CACG	6
71	UCUU	21	AUUC	7	GUUU	5	ACUU	7	AGUG	2	CCUA	6
72	GGCA	21	AAGA	7	UGUA	5	GCUA	7	ACUG	2	CCCC	6
73	AUCC	21	UGCG	6	UUAG	5	AUCU	7	AAGG	2	UAGC	6
74	AUUA	21	GUAG	6	AUAC	5	UACA	7	UGAC	2	UGGG	6
75	CAAA	21	UUGC	6	CAGU	5	AUAC	7	GCGU	2	AUGG	6
76	ACAA	21	UCUU	6	GAAU	5	CUAA	7	CGGC	2	UAGU	5
77	AGCU	21	CCCG	6	AAGA	5	UCCA	7	GAGA	1	UGCG	5
78	UCAG	21	UCCC	6	GACG	5	CUAC	7	GAAG	1	AUAA	5
79	AAAU	20	AGAU	6	GCGG	5	GAGU	6	AAAA	1	UAAG	5
80	AUAA	19	GAUC	6	AAAG	4	CCUU	6	UUUC	1	AAGG	5
81	GAAU	19	GACG	6	CCUU	4	CAAU	6	GACA	1	UUUC	5
82	AUGA	18	GAUU	6	AAGU	4	UGCG	6	UUAG	1	GACA	5
83	CGCA	18	CCGC	6	UCAC	4	AUCA	6	GUCA	1	UUCU	5
84	GCCC	18	UACG	5	UAAG	4	ACAA	6	UUCU	1	GCUA	5
85	AUCG	17	GCCA	5	ACUU	4	UCUC	6	AGCU	1	GGCA	5
86	AUGU	17	UCAC	5	UACA	4	UUAC	6	ACUU	1	GUUA	5
87	AGGA	17	CUAG	5	GGUC	4	UAAG	6	GCUA	1	UAUC	5
88	UAUU	16	GGUA	5	GGUA	4	AGAA	6	AUCU	1	UCUG	5
89	GGAU	16	AAGC	5	CAAA	4	GGCA	6	UACA	1	CCAG	5
90	UGUG	16	CCCA	5	ACAU	4	UCAA	6	AUAC	1	UUGU	5

91	UAAU	15	CUAU	5	ACCG	4	AAGA	6	GGCA	1	GUAG	5
92	UUCA	15	AGAC	5	GUUC	4	CUCU	6	UCAA	1	CUCC	5
93	UGAA	15	CAUU	5	AUCA	3	UGAU	5	GUUA	1	GUCU	5
94	AAGU	15	ACUC	5	UGUU	3	CCAA	5	CUGA	1	CAGA	5
95	GGUA	15	CAGG	5	ACAA	3	GUUA	5	UCAG	1	AUCA	4
96	AACU	14	AACC	4	UGCU	3	CUGA	5	CUAG	1	UGUU	4
97	AAUC	14	UGGU	4	CCAA	3	UCCU	5	UAUG	1	UUGG	4
98	CCCG	14	UUUC	4	AAUG	3	UCCG	5	AUAG	1	CUUU	4
99	UGCC	14	GAUA	4	ACGA	3	CCCC	5	UGGC	1	UGGU	4
100	CUGC	14	AUCC	4	CCUA	3	GUGU	5	UAUC	1	UGCA	4
101	UUGA	13	AGGA	4	UCUG	3	CACA	5	CUGU	1	GUCA	4
102	UCUG	13	GGAU	4	CUUC	3	GAUC	5	UCGG	1	AUCU	4
103	CUGA	13	UGCC	4	AUUG	3	AAGU	4	GUUG	1	CUGU	4
104	CAUG	13	CUGC	4	CUGA	3	UGCU	4	AGUA	1	UCGG	4
105	AAGC	13	GCUU	4	UCCU	3	ACUA	4	CCUC	1	CCUG	4
106	UCCC	13	AUGG	4	CUCA	3	GUAC	4	AGCC	1	CGUG	4
107	UGGC	13	GAGG	4	CUCG	3	UGUA	4	CACC	1	AAGA	4
108	AGAA	12	GUAC	4	UCUA	3	UCAG	4	CAAG	1	GUGU	4
109	GAAC	12	GCCG	4	AGUC	3	CUAG	4	CACG	1	AUGA	4
110	CAGU	12	CCGA	4	AACU	3	UAUG	4	UCUG	1	CUGG	4
111	UGUU	12	GUGC	4	UCCA	3	GCAU	4	CCAG	1	ACCC	4
112	CCAU	12	CUAC	4	CUCU	3	CAGU	4	CUUC	1	AGCA	4
113	GUCC	12	GCUA	3	GGCU	3	CUCC	4	AUUG	1	UGAU	3
114	UCGC	12	GACA	3	GCUG	3	GACG	4	GGUA	1	UAUU	3

115	GAGC	12	UCAA	3	AUUC	3	ACCA	4	CCCU	1	AUUA	3
116	ACCA	11	UGAG	3	ACAC	3	AACG	4	GCUC	1	GAAC	3
117	AUCU	11	UAUG	3	AGAU	3	GCAC	4	UCGA	1	GUUU	3
118	CCUC	11	CAAA	3	CAUG	3	GCCC	4	CUAU	1	AGAA	3
119	UGAU	11	AGCU	3	UGAG	2	AUUC	4	CAGG	1	UGAC	3
120	AUCA	11	GAAC	3	CAAU	2	ACAC	4	GGGU	1	GCGU	3
121	UCCU	11	GAGC	3	AAGC	2	UGAG	3	UGCC	1	UACA	3
122	CCAG	11	ACCA	3	CAAC	2	UGUG	3	CCUA	1	UCAA	3
123	AUAU	10	CCUC	3	AAGG	2	UAGA	3	CCUG	1	UAUG	3
124	GAGU	10	UCCU	3	GUGG	2	UUGA	3	CGUG	1	GUUG	3
125	AGUU	10	AGUU	3	ACUG	2	AUAG	3	GGUC	1	GCCA	3
126	AUAC	10	UUCC	3	GGGU	2	UGGC	3	CCGA	1	GAGG	3
127	ACAC	10	CUCC	3	CAAG	2	UAUC	3	CUUA	1	GUAU	3
128	GUGU	10	CUAA	3	CACG	2	CUGU	3	AGGC	1	CACA	3
129	GACC	10	GCCU	3	UUUC	2	UCGG	3	CGUA	1	GACG	3
130	ACGA	9	ACGC	3	GACA	2	GUUG	3	CGGG	1	ACCA	3
131	UCUA	9	AGUG	3	GCUA	2	AGUA	3	ACGC	1	AACG	3
132	AUUC	9	CGAG	3	UAUC	2	CCUC	3	CGAC	1	UCUA	3
133	AGAU	9	CCUA	3	CGUG	2	AGCC	3	CGCG	1	CUGC	3
134	CUUA	9	GGGG	3	UAUG	2	CACC	3	GGGC	1	GUCC	3
135	GCUU	9	CAAU	3	GUUG	2	ACAG	3	AGAG	1	UCGC	3
136	CUGU	9	ACUG	3	AGCU	2	UCCC	3	GCGC	1	UGGA	3
137	GCAC	9	GGCG	3	AGAG	2	CCCA	3	AGGA	1	AAAU	2
138	GUCG	9	CCCC	2	UCGA	2	GAUG	3	GGGG	1	AGUU	2

139	GAUC	9	ACCC	2	CUUA	2	GUCU	3	CGAU	1	CAAU	2
140	CUUU	8	UGAC	2	GCAU	2	AUGA	3	CCGG	1	UCUU	2
141	UACA	8	UCAU	2	ACCU	2	CUGG	3	UUCG	0	AACC	2
142	CCAA	8	GGCA	2	CGCA	2	UCUA	3	GCCA	0	CCAA	2
143	AUUG	8	AUCG	2	UUCC	2	ACUC	3	GAUU	0	ACUA	2
144	UCCA	8	AUGU	2	GAUG	2	AAUC	3	AACA	0	CCAU	2
145	UUCC	8	UGUG	2	UAGC	2	CCCG	3	UUAU	0	AGCU	2
146	CUCC	8	CAGU	2	CUCC	2	GUGC	3	GCAG	0	AGUA	2
147	CUAA	8	CCAU	2	GCCA	2	GUCG	3	AACU	0	CUUC	2
148	GCCU	8	UGAU	2	ACUC	2	CAUA	3	GAAU	0	AUUG	2
149	ACGC	8	AUCA	2	AUCG	2	AGGU	3	CAAA	0	GGUC	2
150	CCCA	8	ACAC	2	AUGU	2	CGUU	3	CAUC	0	ACGC	2
151	CCAC	8	GCAC	2	UCGU	2	AGUU	2	GAGG	0	CGAC	2
152	UAUA	7	GUCG	2	UUGC	2	UUGG	2	AAUU	0	CGCG	2
153	AAUG	7	CCAC	2	GGAU	2	UGGU	2	GACC	0	GGGC	2
154	CUUC	7	AAUG	2	GAUU	2	UGCA	2	UACC	0	AGAG	2
155	AGUC	7	CAGA	2	GGUG	2	ACGA	2	GGAG	0	AACA	2
156	CUAU	7	AUAG	2	AGAC	2	CAAG	2	UUGU	0	GACC	2
157	CAGA	7	CCCU	2	GCUU	2	CACG	2	CUCG	0	GCAC	2
158	AGGU	7	AACG	2	UACU	2	UCUG	2	UUCC	0	GCCC	2
159	UCUC	7	CUCU	2	AAUC	2	CCAG	2	GUAG	0	ACUC	2
160	ACUA	7	GAUG	2	CCCG	2	CUUC	2	GUAU	0	GAGC	2
161	AUAG	7	UACU	2	AUGC	2	AUUG	2	CUAA	0	AUCG	2
162	CCCU	7	CGAC	2	CGAG	2	GGUA	2	UCCA	0	AUGU	2

163	UACC	7	CGAA	2	CGCU	2	CCCU	2	CUAC	0	GCGG	2
164	CACA	7	CAUA	2	UGUG	1	GCUC	2	AAGA	0	UCGU	2
165	AACG	7	GCUG	2	UGCG	1	UCGA	2	CUCU	0	AGUC	2
166	CGGA	7	CGCG	2	UGGU	1	CUAU	2	UCCU	0	UUGC	2
167	AGUA	6	UGCU	2	UGCA	1	CGAA	2	UCCG	0	GGAU	2
168	UAGU	6	UAUC	2	GCGU	1	CUCA	2	CCCC	0	ACGU	2
169	ACUU	6	UUGG	2	CCAU	1	CGCA	2	GUGU	0	CCGU	2
170	CUCU	6	GCGG	2	GUAC	1	UAGC	2	CACA	0	AAAG	1
171	UCGA	6	UGGG	2	UCAG	1	ACCC	2	GAUC	0	UGAA	1
172	AGAC	6	GUUC	2	CUAG	1	CUGC	2	GCAU	0	AAGU	1
173	AUGC	6	CACG	1	CGGG	1	GUCC	2	CAGU	0	ACAA	1
174	GCGU	6	CUCA	1	CCAG	1	GAGC	2	CUCC	0	AAGC	1
175	ACAG	6	UUAC	1	CUGU	1	AUCG	2	GACG	0	AUAU	1
176	CCGU	6	AACA	1	UCAA	1	ACGG	2	ACCA	0	GUAC	1
177	CAGC	6	CACC	1	AGCC	1	GGCU	2	AACG	0	UGUA	1
178	CACU	6	GUAU	1	GCUC	1	GGUG	2	GCAC	0	UUGA	1
179	AGUG	6	AAAG	1	CCGA	1	AGAU	2	GCCC	0	AUAC	1
180	AUGG	6	CGCA	1	CACC	1	CGUC	2	AUUC	0	CCUC	1
181	CAUU	6	UUCA	1	CUAU	1	CCAU	1	ACAC	0	AGCC	1
182	CGUU	6	UCUG	1	AGGA	1	AGUG	1	ACAG	0	GGUA	1
183	GAGG	5	CUGA	1	CGAU	1	ACUG	1	UCCC	0	CCCU	1
184	AAGA	5	UGGC	1	CCGG	1	CAGG	1	CCCA	0	GCUC	1
185	GACG	5	UGUU	1	ACAG	1	GGGU	1	GAUG	0	UCGA	1
186	GGCU	5	GUCC	1	CUAA	1	UGCC	1	GUCU	0	CCGA	1

187	GUGG	5	UCGC	1	UCCC	1	CCUA	1	AUGA	0	CUUA	1
188	GGGU	5	AUCU	1	GCCU	1	CCUG	1	CUGG	0	GCGC	1
189	GAUG	5	CCAG	1	GUCU	1	CGUG	1	UCUA	0	GAUU	1
190	UACU	5	UCUA	1	CAGA	1	GGUC	1	ACUC	0	AACU	1
191	CGAG	5	CUUA	1	GUGU	1	CCGA	1	AAUC	0	CAAA	1
192	GCUC	5	CUGU	1	ACCC	1	CUUA	1	CCCG	0	CAUC	1
193	GUCU	5	CUUU	1	GUCC	1	AGGC	1	GUGC	0	AAUU	1
194	UGUC	5	UACA	1	GCAC	1	ACCU	1	GUCG	0	UCCA	1
195	UAGA	5	UCCA	1	CCGU	1	AUCC	1	CAUA	0	CUCU	1
196	CCUG	5	AGGU	1	GCCG	1	UGGG	1	AGGU	0	ACGG	1
197	CGAC	5	UCUC	1	CAGC	1	CAGA	1	CGUU	0	GGCU	1
198	CGAA	5	UACC	1	GUGC	1	AGCA	1	CGAA	0	GGUG	1
199	AGCG	5	UAGU	1	GUCG	1	UCGC	1	CUCA	0	ACAU	1
200	CAUA	5	AUGC	1	AGGU	1	AUGU	1	CGCA	0	AGAC	1
201	CCUA	4	ACAG	1	UGUC	1	GCGG	1	UAGC	0	GCUU	1
202	GCUG	4	CAGC	1	CACU	1	UCGU	1	ACCC	0	GCCG	1
203	CGUG	4	CGUU	1	UAGA	0	AGUC	1	CUGC	0	GCUG	1
204	UAGC	4	GGCU	1	UCUC	0	ACAU	1	GUCC	0	CAGC	1
205	CGCU	4	GCUC	1	UGAC	0	AGAC	1	GAGC	0	GGAC	1
206	UGCA	4	UGUC	1	ACUA	0	GCUU	1	AUCG	0	GACU	1
207	CGUA	4	UAGA	1	AGUG	0	GCCG	1	ACGG	0	CAUU	1
208	CGCG	4	AGCG	1	CGGC	0	GCUG	1	GGCU	0	UACU	1
209	GGGC	4	UAGC	1	CAGG	0	CAGC	1	GGUG	0	AGCG	1
210	AGGC	4	CGCU	1	AUAG	0	GGAC	1	AGAU	0	GGCC	1

211	GGGG	4	UGCA	1	UGCC	0	GACU	1	CGUC	0	GGUU	1
212	UGGA	4	UGGA	1	CGUA	0	CCGC	1	ACCU	0	CGGA	1
213	UCGG	3	UGUA	1	UGGC	0	AUGC	1	AUCC	0	AUUU	0
214	UAGG	3	CGAU	1	CCUG	0	UGUC	1	UGGG	0	UCUC	0
215	UGUA	3	CGGC	1	ACGC	0	ACCG	1	CAGA	0	UGCU	0
216	UGCU	3	GCGC	1	CGAC	0	UAGG	0	AGCA	0	AAUG	0
217	CAAU	3	GGAC	1	CGCG	0	AAGC	0	UCGC	0	GUGG	0
218	AAGG	3	GGCC	1	GGGC	0	AAGG	0	AUGU	0	ACGA	0
219	ACUG	3	UCGU	1	CCCU	0	UGAC	0	GCGG	0	AGUG	0
220	UAUC	3	GACU	1	GCGC	0	GCGU	0	UCGU	0	ACUG	0
221	AGAG	3	CGUC	1	AGGC	0	CGGC	0	AGUC	0	CGGC	0
222	ACCU	3	CCUU	1	GGGG	0	CGUA	0	ACAU	0	CUGA	0
223	GAUU	3	CCGG	1	GCAG	0	CGGG	0	AGAC	0	CACC	0
224	GUAC	3	CAUC	1	CGAA	0	ACGC	0	GCUU	0	CUAU	0
225	CGAU	3	GGUU	1	UACC	0	CGAC	0	GCCG	0	AGGC	0
226	GCCG	3	GGUG	1	CCCA	0	CGCG	0	GCUG	0	AGGA	0
227	CGGC	3	AGCC	0	CCCC	0	GGGC	0	CAGC	0	GGGG	0
228	GCGC	3	ACGU	0	UGGG	0	AGAG	0	GGAC	0	CGAU	0
229	GGAC	3	CGGU	0	AUGG	0	GCGC	0	GACU	0	CCGG	0
230	GGCC	3	UUGU	0	CACA	0	AGGA	0	CCGC	0	CUAC	0
231	CCGC	3	UCAG	0	AACG	0	GGGG	0	AUGC	0	GAUC	0
232	UUGG	2	GCCC	0	CUGC	0	CGAU	0	UGUC	0	AUUC	0
233	GCGG	2	GUGU	0	UCGC	0	CCGG	0	ACCG	0	ACAC	0
234	GUUG	2	GACC	0	UGGA	0	GCCU	0	GCCU	0	AAUC	0

235	UCGU	2	AUUG	0	GACC	0	AUGG	0	AUGG	0	CCCG	0
236	CGGG	2	AGUC	0	GCCC	0	UGGA	0	UGGA	0	GUGC	0
237	CCGA	2	ACUA	0	GAGC	0	UUGC	0	UUGC	0	GUCG	0
238	UGGG	2	CACA	0	CAUC	0	GGAU	0	GGAU	0	CAUA	0
239	GACU	2	CGGA	0	ACGG	0	ACGU	0	ACGU	0	AGGU	0
240	CGUC	2	UCGA	0	GGAC	0	CCGU	0	CCGU	0	CGUU	0
241	AGGG	2	GCGU	0	GACU	0	CAUU	0	CAUU	0	AGAU	0
242	CUGG	1	CCGU	0	CAUU	0	UACU	0	UACU	0	CGUC	0
243	CCUU	1	CACU	0	AGCG	0	AGCG	0	AGCG	0	CCGC	0
244	GGUC	1	GGGU	0	GGCC	0	GGCC	0	GGCC	0	AUGC	0
245	ACCG	1	CGUA	0	CGGA	0	GGUU	0	GGUU	0	UGUC	0
246	ACUC	1	GGGC	0	CUAC	0	CGGA	0	CGGA	0	ACCG	0
247	CCGG	1	AGGC	0	GAUC	0	CAUG	0	CAUG	0	CAUG	0
248	GUGC	1	UCGG	0	CAUA	0	CGCC	0	CGCC	0	CGCC	0
249	CAUC	1	UAGG	0	CGUU	0	CGAG	0	CGAG	0	CGAG	0
250	ACGG	1	AAGG	0	CGUC	0	GGCG	0	GGCG	0	GGCG	0
251	GGCG	1	AGAG	0	CCGC	0	CCAC	0	CCAC	0	CCAC	0
252	GGUU	0	CGGG	0	CGCC	0	GUUC	0	GUUC	0	GUUC	0
253	GUUC	0	AGGG	0	GGCG	0	CGCU	0	CGCU	0	CGCU	0
254	GGUG	0	CUGG	0	CCAC	0	CGGU	0	CGGU	0	CGGU	0
255	CAGG	0	GGUC	0	CGGU	0	CACU	0	CACU	0	CACU	0
256	CUAC	0	ACCG	0	AGGG	0	AGGG	0	AGGG	0	AGGG	0
uncovered sequence #		5		30		54		41		116		44

### Appendix S1. Calculation of the Spearman's rank correlation coefficient.

The original ranking of the 34 sequences tested, based on normalized NGS data, was re-designated as  $\mathbf{d}_1$  and ranged from 1 to 34. A new ranking, referred to as  $\mathbf{d}_2$ , was established based on the observed yield of loop-closing ligation. The differences between the two rankings were calculated using the formula  $\mathbf{d}_i = \mathbf{d}_1 - \mathbf{d}_2$ , where  $i$  ranges from 1 to 34. Consequently, the Spearman's rank correlation coefficient was calculated to be  $\rho = 0.84$ , as determined by the following equation and data:

$$\rho = 1 - \frac{6 \sum d_i^2}{n(n^2 - 1)}$$

NGS-rank original	reference rank, $\mathbf{d}_1$	Observed yields of loop-closing ligation	Loop-closing rank, $\mathbf{d}_2$	$\mathbf{d}_i^2 = (\mathbf{d}_1 - \mathbf{d}_2)^2$
UGCG-1	1	60.7	3	0
UUCG-3	2	63.0	1	0
UUUG-4	3	43.3	6	9
UACG-5	4	53.7	4	0
UCCG-6	5	63.0	2	4
UUAG-8	6	53.3	5	1
UUGG-11	7	32.3	13	36
UCGC-12	8	26.0	16	64
UUGC-14	9	23.0	20	121
UCGG-15	10	42.0	7	9
CCUG-16	11	31.7	14	9
CAGG-17	12	21.3	21	81
UCUG-18	13	40.3	9	16
CUCG-21	14	41.0	8	36
GGGA-22	15	18.7	22	49
CUGG-23	16	18.7	23	49
CAAG-25	17	33.0	12	25
UGGG-27	18	24.3	19	1
GAGA-32	19	26.0	17	4
GUGA-34	20	25.3	18	4
CCAG-35	21	37.3	11	100
UAAC-38	22	18.7	24	4
UGGC-39	23	13.7	26	9
GCGA-44	24	38.0	10	196

UUCA-69	25	9.0	27	4
UCCA-75	26	7.3	28	4
UUUA-86	27	16.0	25	4
GAAA-87	28	31.3	15	169
UGGU-93	29	3.0	29	0
GCCC-240	30	0.4	30	0
GACU-245	31	0.1	34	9
AAAU-252	32	0.2	31	1
AAGC-253	33	0.1	33	0
AACC-256	34	60.7	32	4

## Appendix S2. Hypergeometric test.

Results are represented in main text and Figure S13. The comparison of overlaps between the top 40 sequences from loop-closing ligation and the top 40 biological tetraloop sequences supported by ...n be effectively described using the hypergeometric distribution. For illustration, consider the following scenario: a bag contains 256 apples, 40 of which are green and 216 are red. If one were to randomly draw 40 apples from the bag without replacement, what would be the expected number of green apples? The probability of this outcome can be modelled by the following

$$p_X(k) = \Pr(X = k) = \frac{\binom{K}{k} \binom{N-K}{n-k}}{\binom{N}{n}},$$

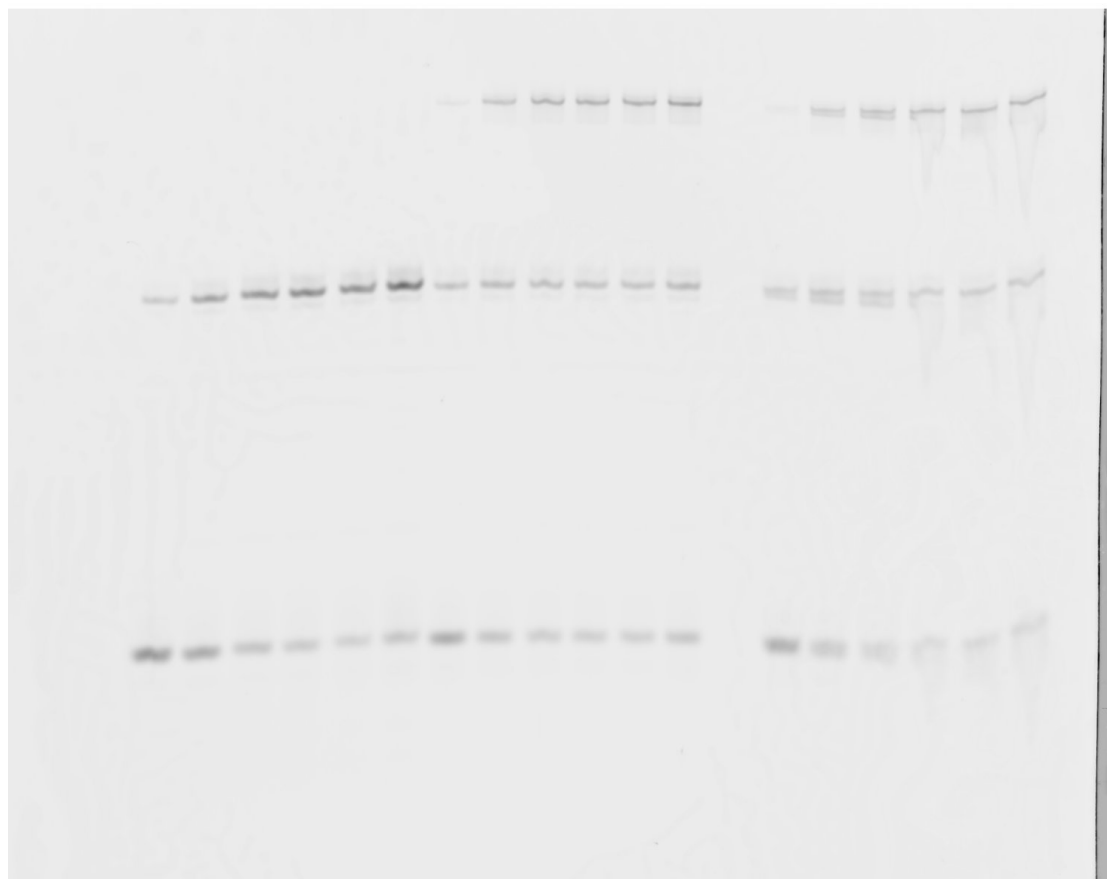
equation:

where  $N$  (256 apples) represents the total population size,  $K$  is the number of successes (40 green apples) in the population,  $n$  is the number of draws (20, 30, 40, or 50 as used in this study), and  $k$  is the number of observed successes (green apples).

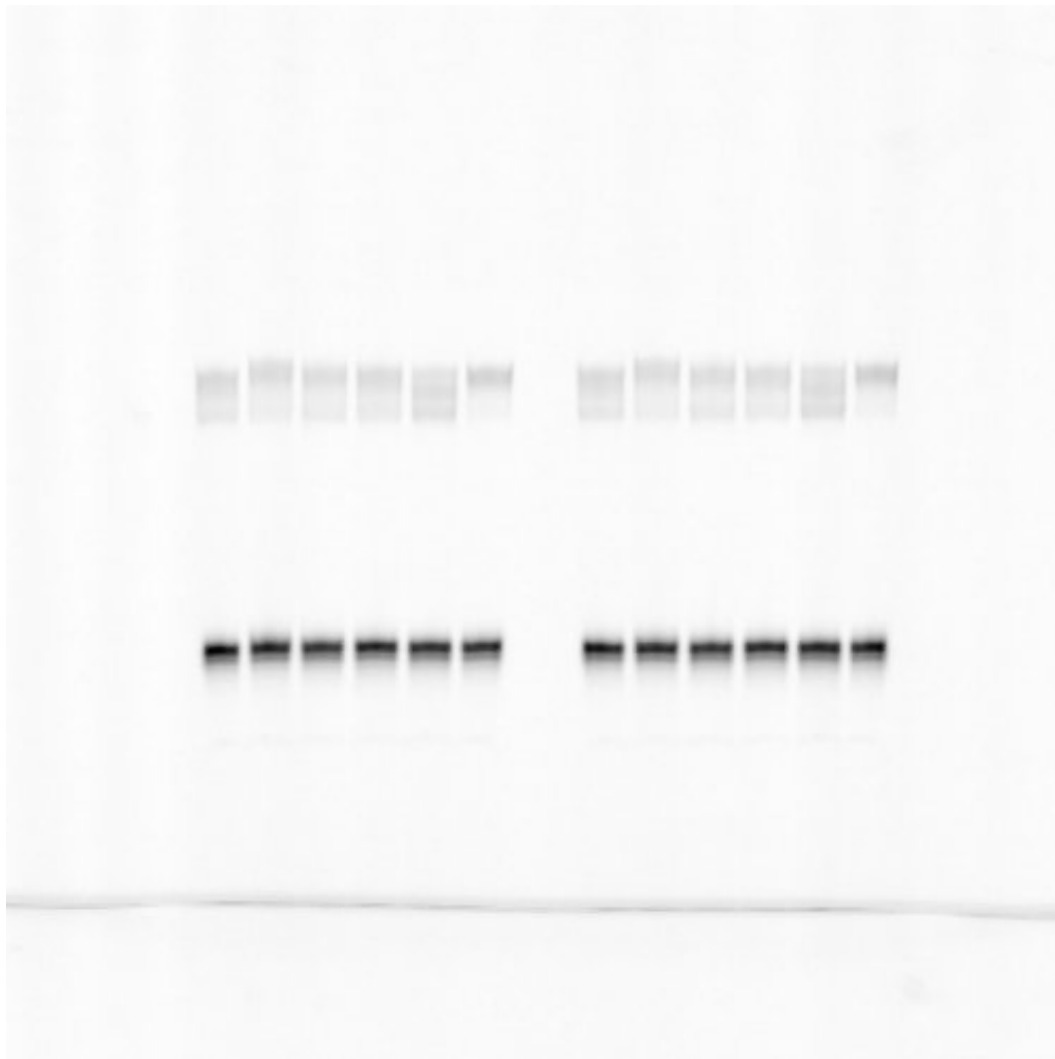
The hypergeometric test is employed to measure the statistical significance of over-representation or under-representation. An observed number of successes greater than expected indicates over-representation, while a number lower than expected suggests under-representation. The hypergeometric p-value is calculated as the probability of observing  $k$  or more successes in a test for over-representation (as shown Figure S13). Conversely, for under-representation, the hypergeometric p-value is calculated as the probability of observing  $k$  or fewer successes.

**Uncropped, full-size gel images, with no contrast changing, presented in the manuscript.**

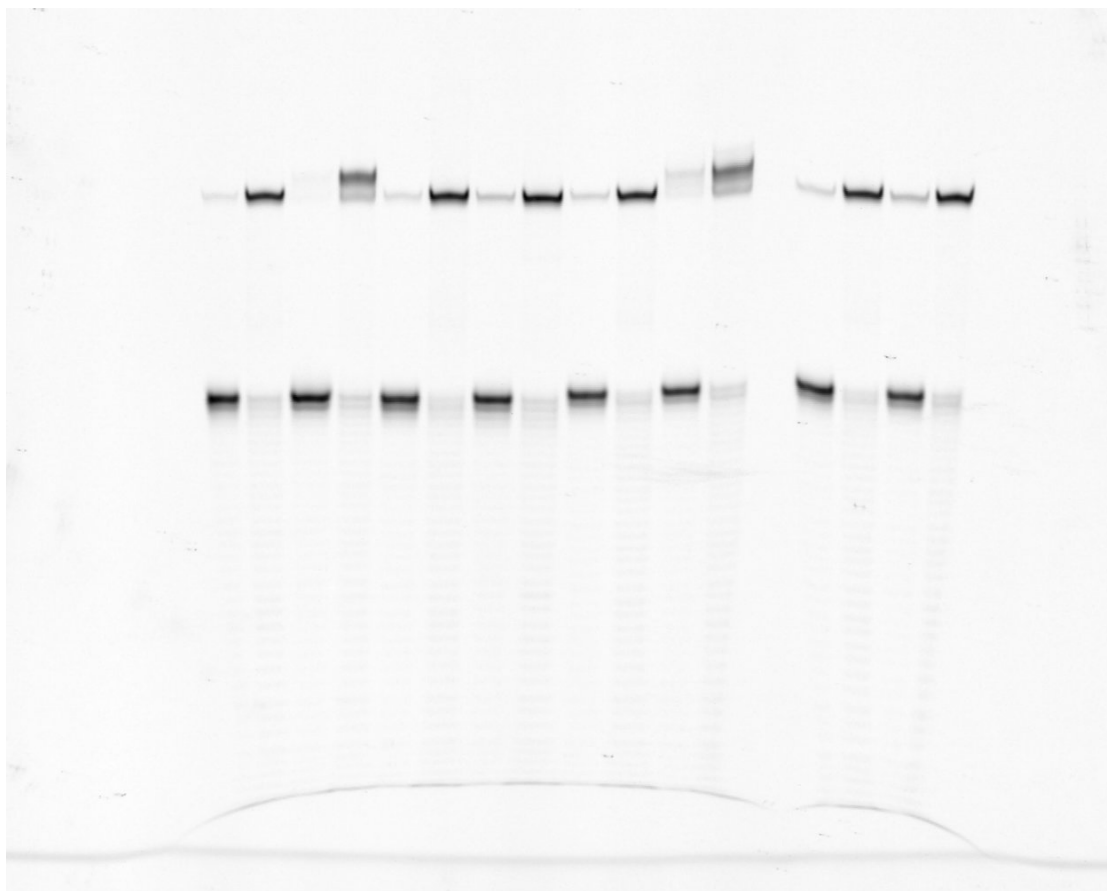
Gel image for Figure 5D:



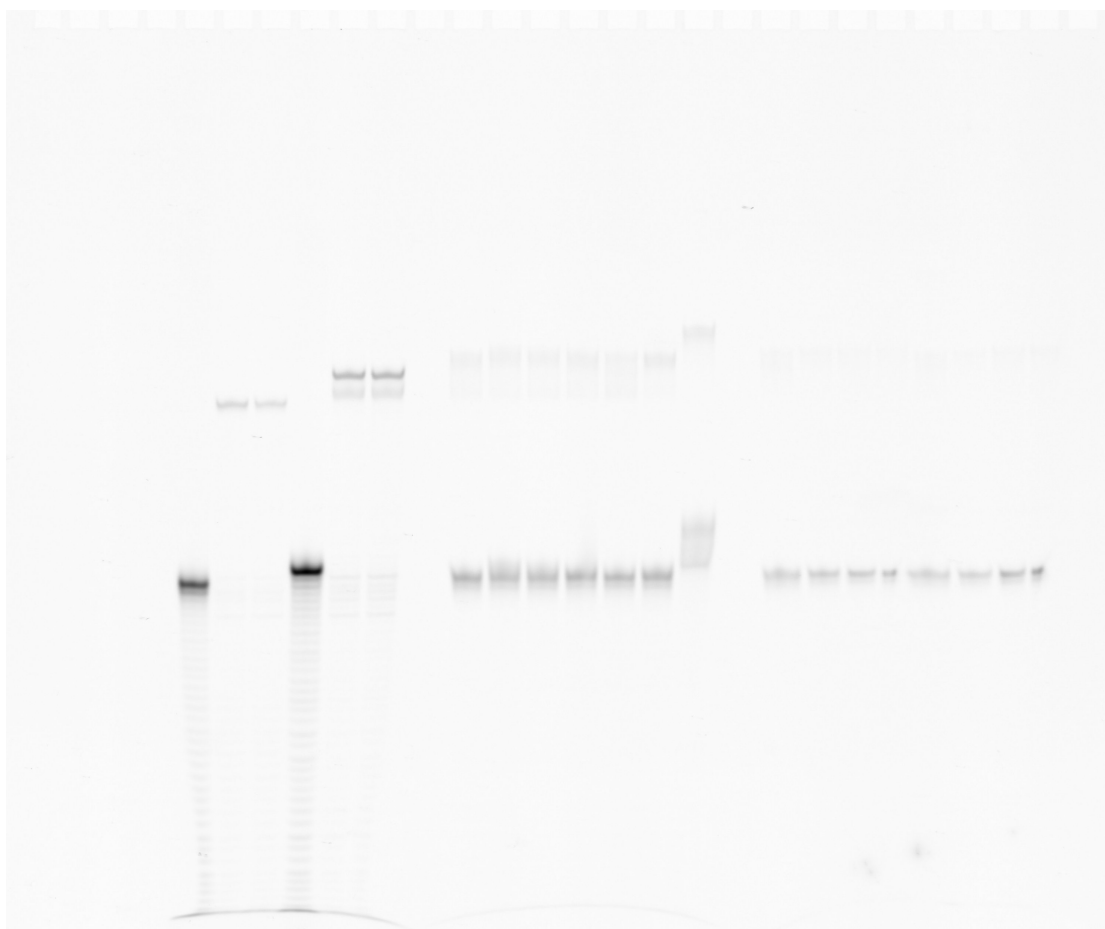
Gel image for Figure S2:



Gel image for Figure S3:



Gel image for Figure S5:



Gel image for Figure S10:

