Supplementary Information

The Role of Sugar-backbone Heterogeneity and Chimeras in the Simultaneous Emergence of RNA and DNA

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| Content                                                                 | Page |
|------------------------------------------------------------------------|------|
| General experimental                                                   | 3    |
| Preparation of the (3′→2′) threofuranosyl phosphoramidite             | 4    |
| Synthesis and UV-Tm studies of TNA-RNA chimeric oligonucleotide sequences | 4-17 |
| Non-enzymatic Ligation reaction with TNA-RNA sequences                 | 18-35|
| Synthesis and UV-Tm studies of DNA-RNA chimeric oligonucleotide sequences | 36-45|
| Non-enzymatic Ligation reaction with DNA-RNA sequences                 | 46-100|
| Effect of stepwise dilution on replication cycle                       | 101-103|
| Attempted cross-catalytic self-replication                             | 104  |
| Enzymatic ligation reaction                                            | 105-113|
| Supplementary References                                               | 114  |
General Experimental:

Reagents and solvents were purchased from Sigma-Aldrich, VWR International, Fischer Scientific, Acros Organics. DNA and RNA CPG and phosphoramidites were purchased from Glen Research and ChemGenes. T4 DNA ligase and T4 RNA ligase 2 enzymes were purchased from New England Biosciences. TNA phosphoramidites were synthesized in the laboratory using literature reported method. TNA & TNA-RNA chimeric sequences were synthesized using "Expedite synthesizer" following standard reported protocol. TNA ligands were utilized from the laboratory inventory made by Wu et al. DNA, RNA and DNA-RNA chimeric oligonucleotides were either synthesized on an Expedite 8909 Nucleic Acid Synthesis System (PerSeptive Biosystems), or purchased from IDT and used without further purification. 3'-Amino-dT CPG and Solid Chemical Phosphorylation Reagent II were purchased from Glen Research and used for TNA-RNA chimeric, DNA and RNA ligand synthesis. Thin layer chromatography (TLC) were performed on silica gel 60 Å F254 from Angela Technologies and visualized by UV lamp and/or a stain solution of phosphomolybdic acid (PMA) in ethanol. Flash Chromatography was performed on Biotage Isolera. NMR were recorded at 298 K: Bruker DRX-600 or AV-600 (600 MHz for 1H and 150 MHz for 13C). 31P-NMR spectra were acquired using a Bruker DPX-400; Desalting of oligonucleotides were carried out using DEAE-A-25-sephadex, 40-120 mesh, HCO3⁻ form, 2.5 x 3.5 cm column with TEAB buffer of pH 7. Quantification of oligonucleotide was done using NanoDrop 2000c spectrometer. Anion Exchange Chromatography (AEC) was performed with AKTA purifier (900) with UNICORN system control using 260 nm on a DNApacTMPA200 column with a flow rate of 1 mL/min using (0.8 mL/min for ligation reactions) either condition 1 (Buffer A: 10 mM Na2HPO4, pH 11 and Buffer B: 10 mM Na2HPO4, 1 M NaCl, pH 11) or condition 2 (Buffer A: 5 mM Tris base, pH 8.2 and Buffer B: 5 mM Tris base, 330 mM NaClO4, pH 8.2). The gradient is specified under each AEC profile. The samples were filtered through 0.22 μm syringe tip filter units (Milliex-GV; PVDF). MALDI-TOF was conducted using Voyager DE-Pro Workstation, made by Applied Biosystems in Prof. Steven Dowdy lab at University of California, San Diego with trihydroxyacetophenone (THAP) as the matrix with ammonium citrate added to each sample. ZipTip used for desalting for MALDI-TOF was purchased from Merck Millipore limited. Thermal duplex stability was measured using a Cary 100 UV-Vis spectrophotometer, at 260 nm with a heating/cooling rate of 1 °C per minute. The pH was measured using ACCuMET BASIC AB15 Plus pH meter.
Preparation of the (3′→2′)threofuranosyl phosphoramidite.

Our approach of synthesizing (3′→2′)-threofuranosyl phosphoramidites was parallel to the one developed by Chaput et al.\(^1\) (as depicted in Supplementary Figure 1). All the compounds were characterized by \(^1\)H, \(^13\)C and \(^31\)P NMR and are identical to those reported in the literature.\(^{1,2}\)

![Chemical diagram of the synthesis process](image)

Supplementary Figure 1. Synthesis of (3′→2′) threofuranosyl phosphoramidite monomer

Synthesis of oligonucleotide\(^2\):

Oligomer synthesis was conducted on a 1 \(\mu\)mol scale with universal solid support or with commercially available solid support loaded with RNA nucleoside (500 Å, Glen Research Q PE Biosystems) by following the literature method and purified using anion exchange chromatography and desalted using TEAB buffer.\(^2\) The purified oligonucleotide was re-dissolved in nuclease free water and the concentration of the stock solution was recorded using Nanodrop at 260 nm wavelength.
### Supplementary Table 1: AEC and MALDI-TOF data of TNA-RNA chimeric oligonucleotide sequences.

| Entry | Sequence | retention time [min]a | MALDI-TOF-MS (Calculated) [M + H]+ | MALDI-TOF-MS (Observed)b |
|-------|----------|------------------------|-------------------------------------|--------------------------|
| 1     | 5′-(Au)₈-2′ (3′) | 24.4                   | 4777                                | 4778                     |
| 2     | (5′)₃′-(aU)₈-3′ | 24.7                   | 4777                                | 4777                     |
| 3     | 5′-As₈-2′(3′)   | 23.6                   | 4777                                | 4775                     |
| 4     | (5′)₃′-as₈-3′   | 24.3                   | 4777                                | 4778                     |
| 5     | 5′-AUUAUAUAuUAUAUAU-3′ | 25.2                   | 4988                                | 4982                     |
| 6     | 5′-AUUAUAUAuUAUAUAU-3′ | 24.4                   | 4958                                | 4957                     |
| 7     | 5′-AUUAUAUAuUAUAUAU-3′ | 21.8                   | 4988                                | 4984                     |
| 8     | (5′)₃′-aAaAtTtAtTaTtA-3′ | 23.5                   | 4893                                | 4890                     |
| 9     | 3′-TtTtAaTtAtAaTtA-3′ (5′) | 24.2                   | 4893                                | 4891                     |
| 10    | (5′)₃′-aAaAuUuAuAuUuA-3′ | 24.3                   | 4778                                | 4775                     |
| 11    | 3′-UuUuAaAuAuUuAaAu-3′(5′) | 24.3                   | 4778                                | 4772                     |
| 12    | (5′)₃′-aaaaTTTAaTtAttA-3′ | 23.5                   | 4863                                | 4860                     |
| 13    | 3′-TTTTtaaATtAaTAtt-3′ (5′) | 23.5                   | 4923                                | 4920                     |
| 14    | (5′)₃′-aaagTTTAaTtAttC-3′ | 24.5                   | 4865                                | 4863                     |
| 15    | 3′-TTTTCaatATGcGAt-3′ (5′) | 29.3                   | 4925                                | 4926                     |
| 16    | (5′)₃′-aaaaTTTATNH₂-3′ | 10.9                   | 2727                                | 2728                     |
| 17    | 5′-PO₃²⁻-AttAttA-3′ | 11.4                   | 2166                                | 2167                     |
| 18    | 5′-AAAATTTATNH₂-3′ | 12.2                   | 2847                                | 2848                     |
| 19    | 5′-PO₃²⁻-ATTATTATTA-3′ | 12.7                   | 2286                                | 2287                     |

Oligonucleotides were purified using AEC. a 0 to 100% buffer B gradient using 5 mM Tris base, pH 8.2 as Buffer A and 5 mM Tris base, 330 mM NaClO₄, pH 8.2 as Buffer B; peak purity (260 nm) ≥ 95%. b) MALDI was recorded using THAP matrix; a,u,t,g,c = TNA; A,U,T,G,C = RNA; T = DNA.

### Biophysical Analysis

**Duplex Stability of TNA-RNA Chimeras:** From a stock solution of the oligonucleotide, appropriate amount was pipet off and lyophilized to dryness. To that dried oligonucleotide mixture 400 µL of phosphate buffer (with either 150 mM or 1 M NaCl as indicated in the table) was added and the samples were thoroughly degassed by vacuum and ultrasonication. Then, the UV-monitored duplex stability was carried out using a Cary 100 UV-Vis, at 260 nm frequencies with a heating/cooling rate of 1 ºC per minute. Melting temperatures (Tm) were calculated using the Van’t Hoff two-state model and averaged from multiple heating/cooling cycles.
**Supplementary Table 2:** UV-Tm thermal stability of TNA-RNA chimeric sequences

| Entry | Sequence | Conc.(total) | Tm (°C) | ΔTm (°C) |
|-------|----------|--------------|---------|----------|
| 1.    | (5’)3’-(aT)_8-3’ | 10 µM | 25.8† | -33 |
| 2.    | (5’)3’-(tA)_8-3’ | 10 µM | 51 | -7.8 |
| 3.    | 5’-(At)_8-2’(3’) | 10 µM | 50.1† | -8.7 |
| 4.    | 5’-(Au)_8-2’(3’) | 10 µM | 13.2 | -40.4 |
| 5.    | (5’)3’-(aU)_8-3’ | 10 µM | 9.5 | -44.1 |
| 6.    | 5’-A_A_8-2’(3’) | 10 µM | <5† | -40.9 |
| 7.    | (5’)3’-asT_8-3’ | 10 µM | 61.3† | +20.4 |
| 8.    | 5’-A_A_8-2’(3’) | 10 µM | <5 | -40.9 |
| 9.    | (5’)3’-asU_8-3’ | 10 µM | 38.6 | -2.3 |
| 10.   | 5’-AUAUAUAuAUAUAUAU-3’ | 10 µM | 43.3 | -10.3 |
| 11.   | 5’-AUAUAUAuAUAUAUAU-3’ | 10 µM | 34.2 | -19.4 |
| 12.   | 5’-AUAUAUAUAuAUAUAUAU-3’ | 10 µM | 46.4 | -7.2 |
| 13.   | 5’-AAAATTTTAT A TTATTA-3’+ 3’-TTTTAATATAATAAT-5’ | (2+2) µM | 43.8** | -5.3 |
| 14.   | 5’-AAAAT T t At ATTAT TA-3’+ 3’-T T TAAATATAATAAT-5’ | (2+2) µM | 33.5** | -15.4 |
| 15.   | 5’- AAA ATA t t A t At t A-3’+ 3’-TT T TAAATATAATAAT-5’ | (2+2) µM | <10** | - |
| 16.   | (5’)3’-aAaTtAtTaTtA-3’ | 10 µM | - | - |
| 17.   | (5’)3’-tAaTaAtAtTaTt-3’ | 10 µM | - | - |
| 18.   | (5’)3’-aAaAtT tAtT aT-3’+ 3’-T tT tAaAtAtAaTaAt-3’ (5’) | (10+10) µM | - | - |
| Entry | Sequence                                                                 | Conc.(total) | Tm (°C)† | ΔTm (°C) |
|-------|--------------------------------------------------------------------------|--------------|-----------|-----------|
| 19    | (5′)3′-aAaAuUuAuUuAuUuA-3′                                              | (5+5) µM    | -         | -         |
|       | 3′-UuUuAaAuAaAaAu-3′(5′)                                               |              |           |           |
| 20    | (5′)3′-aAaAtT t AtA tT aTtA-3′+                                          | (2+2) µM    | <5        | -         |
|       | 3′-TTTTAAATATAATAAT-5′                                                  |              |           |           |
| 21    | 5′-AAAAATTTATATTATTTA-3′+                                               | (2+2) µM    | <5        | -         |
|       | 3′-TtT t AaAtA tAa TaAt-3′ (5′)                                         |              |           |           |

**RNA series for comparison**

| Entry | Sequence | Conc. | Tm (°C) |
|-------|----------|-------|---------|
| 22    | 5′-(AU)8-3′ | 10 µM | 53.6    |
| 23    | 5′-A8U8-3′  | 10 µM | 40.9    |
| 14    | 5′-AAAAATTTATATTATTA-3′+                                               | (2+2) µM    | 48.9**  |
|       | 3′-TTTTAAATATAATAAT-5′                                                 |              | 59.5**  |

* 1M NaCl, 10 mM Na₂HPO₄, 100 µM EDTA pH 7.2 buffer; † Reference 2; ** Reference 4; # ΔTm is w.r.t. corresponding RNA sequences; A,U,T = RNA, a,u,t = TNA, \( A,T \) = DNA.
**Strong-Weak base pairing trend in TNA-RNA chimeras**

Supplementary Figure 2. A dichotomous thermal stability behavior of self-complementary hexadecameric TNA-RNA chimeric duplexes. Figure (i), left side shows that in an alternate TNA-RNA chimeric sequence TNA-pyrimidine and RNA-purine gave stronger duplexes (Table 2, Entry 1-5) while in Figure ii (right side) a block TNA-RNA chimeric sequence TNA-purine and RNA-pyrimidine gave stronger (Supplementary Table 2, Entry 6-9) duplexes. a,t,u = TNA and A,T,U = RNA.
Supplementary Figure 3. Melting curves for self-complementary alternate TNA-RNA chimeric sequences (Supplementary Table 2, Entry 2,4-5) in 10 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1 M NaCl, 100 µM EDTA at pH 7.2; a,t,u = TNA and A,T,U = RNA.

Supplementary Figure 4. Melting curves for self-complementary alternate TNA-RNA chimeric sequences (Supplementary Table 2, Entry 8-9); in 10 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1 M NaCl, 100 µM EDTA at pH 7.2; a,u = TNA and A,U = RNA.
**Supplementary Figure 5.** Melting curves indicating the effect of TNA monomer insertion into a self-complementary RNA sequence (Supplementary Table 2, Entry 10-12) in 10 mM Na$_2$HPO$_4$, 1 M NaCl, 100 µM EDTA at pH 7.2; a,u = TNA and A,U = RNA.

**Supplementary Figure 6.** Melting curves of alternate TNA/RNA non-self-complementary chimeric sequences. (Supplementary Table 2, Entry 16-18) in 10 mM Na$_2$HPO$_4$, 1 M NaCl, 100 µM EDTA at pH 7.2; a,u = TNA and A,U = RNA.
**Supplementary Table 3:** Duplex thermal stability of TNA/RNA chimeric sequences and it’s complementary strands

| Entry | Sequence | Description | T<sub>m</sub> (°C)<sup>a</sup> | ΔT<sub>m</sub> (°C)<sup>#</sup> |
|-------|----------|-------------|-------------------------------|--------------------------------|
| 1     | (5′)3′-aaaaTTTAtAttA-3′ | TRNA | - | - |
| 2     | (5′)3′-tAAtAAtaaTTTT-3′ | TRNA | - | - |
| 3     | (5′)3′-aaaaTTTAtA t A t A-3′+3′-TTTTaatAtAAAtA-3′ (5′) | TRNA | <20* | > -39.5 |
|       | TRNA | <20″* | > -39.5 |
| 4     | (5′)3′-aaaa TTTAtA t A t A-3′+3′-TTTTaatAtAAAtA-3′ (5′) | TRNA | 20.2 | -39.3 |
|       | DNA | 25 | -34.5 |
| 5     | 5′-AAAAATTTATATTATT-3′+3′-TTTTa aat AtAAAtA-3′ (5′) | DNA | 40.5 | -19.0 |
|       | TRNA | 42 | -17.5 |
| 6     | 5′-AAAAATTTATATTATT-3′+3′-TTTT T a a A t AaAAtA-3′ (5′) | RNA | 35.5 | -21.2 |
|       | TRNA | 31.6 | -27.9 |
| 9     | 3′-a a a a t a t t A t t t a-2′+3′-TTTTaatAtAAAtA-3′ (5′) | TNA | 25.1 | -34.4 |
|       | TRNA | 38.3 | -24.0 |
| 10    | (5′)3′-a a T T T A t A t A t A-3′+3′-TTTTaatAtAAAtA-5′ | RNA | 25.1 | -34.4 |
| 11    | (5′)3′-a a a A t T A t A t A-3′+3′-TTTTaatAtAAAtA-5′ | RNA | 31.6 | -27.9 |
| 12    | 5′-AAAAUUUAUAUAAUAUAUAUAUAUA-3′+3′-TTTT T a a A t AaAAtA-3′ (5′) | RNA | 25.1 | -34.4 |
| Entry | Sequence | Description | $T_m$ ($^\circ$C)$^a$ | $\Delta T_m$ ($^\circ$C)$^\#$ |
|-------|----------|-------------|----------------------|------------------------|
| 13    | (5')3'-a a atTTTAt cGc tA-3'+ 3'-TTTCa a atAGcGAt-3' (5') | TRNA TRNA | <30* | > - 35.5 |
| 14    | (5')3'-a a a gTTT A t A t c Gc t A-3'+ 3'-UUUCAAUAUAAGCGAU-5' | TRNA RNA | <45* | > - 25.5 |
| 15    | 5'-AAAGUUUAUAUCGCUA-3'+ 3'-TTT Ca a a t A t A Gc GAt-3' (5') | RNA TRNA | 52.5 | -13 |

**RNA series for comparison**

| Entry | Sequence | Description | $T_m$ ($^\circ$C) | $\Delta T_m$ ($^\circ$C)$^\#$ |
|-------|----------|-------------|-------------------|------------------------|
| 16    | 5'-AAAATTTATATTATTA-3' + 3'-TTTTAAATATAATAAT-5' | RNA RNA | 59.5 | 48.9§ |
| 17    | 5'-AAAGUUUAUAUCGCUA-3'+ 3'-UUUCAAUAUAAGCGAU-5' | RNA RNA | 65.5 | |

$a = 1$ M NaCl, 10 mM Na$_2$HPO$_4$, 100 $\mu$M EDTA pH 7.2 buffer; $b = 150$ M NaCl, 10 mM Na$_2$HPO$_4$, 100 $\mu$M EDTA pH 7.2 buffer; * No clear sigmoidal melting curve was observed; † = (10+10) $\mu$M; $^\# \Delta T_m$ w.r.t. parent RNA duplex (entry 16-17); § Reference 4; a,t,u,g,c = TNA and A,T,U,G,C = RNA.
**Supplementary Figure 7.** Melting curves of TNA-RNA chimeric sequences (single strands and duplexes) (Supplementary Table 3, Entry 1-3) in 10 mM Na₂HPO₄, 1 M NaCl, 100 µM EDTA at pH 7.2 buffer; a,t = TNA and A,T = RNA.
Supplementary Figure 8. Melting curves of TNA-RNA chimeric sequences and their complementary heterogeneous and homogeneous duplex strands in 10 mM Na$_2$HPO$_4$, 1 M NaCl, 100 µM EDTA at pH 7.2 buffer. It shows that the TNA-RNA chimeric strand prefers complementary homogeneous strands over its TNA-RNA heterogeneous counterpart (Supplementary Table 3, Entry 3-6,9-10). a,t = TNA, A,T = RNA; A,T =DNA.
Supplementary Figure 9. Melting curves of TNA-RNA chimeric sequences indicates the preference of heterogeneous TNA-RNA chimeric sequence for the homogeneous RNA sequence (green) over the heterogeneous TNA-RNA sequence (orange) in 10 mM Na₂HPO₄, 1 M NaCl, 100 µM EDTA at pH 7.2 buffer. The orange line indicates the UV-Tm curve of complementary TNA-RNA chimeric sequence and the green line indicates the UV-Tm curve of a mixture containing two complementary TNA-RNA chimeric sequence and homogeneous RNA sequence (complementary to bottom strand). The green line signifies that even from a homogeneous and heterogeneous mixture, the chimeric sequence selects complementary homogeneous sequence. a,t = TNA, A,T = RNA.
Supplementary Figure 10. Melting curves comparison of the duplexes formed from the two possible combinations of TNA/RNA chimeric sequences with their corresponding complementary homogeneous RNA (A,U) sequences (Supplementary Table 3, Entry 1-2) in 10 mM Na$_2$HPO$_4$, 1 M NaCl, 100 µM EDTA at pH 7.2 buffer; a,t = TNA and A,T,U = RNA.

Supplementary Figure 11. Melting curves comparison of the duplexes formed from the TNA-RNA (A,T/U,G,C) chimeric sequences with their complementary homogeneous RNA (A,U) sequences (Supplementary Table 3, Entry 13-15) in 10 mM Na$_2$HPO$_4$, 1 M NaCl, 100 µM EDTA at pH 7.2 buffer; a,t,g,c = TNA and A,T,U,G,C = RNA.
**Supplementary Figure 12.** Melting curves of TNA-RNA chimeric sequences and its complementary homogeneous RNA strand with phosphodiester linkage (green) and with corresponding dT phosphoramidate linkage (red). It shows that the duplex with full RNA and full RNA with single dT phosphoramidate linkage have similar stability. (Supplementary Table 3, Entry 6-7) in 10 mM Na$_2$HPO$_4$, 1 M NaCl, 100 µM EDTA at pH 7.2 buffer.

**Supplementary Figure 13.** Melting curve of homogeneous RNA and its complementary homogeneous RNA strand with single DNA T phosphoramidate linkage (Supplementary Table 3, Entry 8) in 10 mM Na$_2$HPO$_4$, 1 M NaCl, 100 µM EDTA at pH 7.2 buffer.
General experimental procedure for the template directed non-enzymatic ligation reaction. 
0.1 mM template and 0.2 mM of each ligands were pipetted in to a 0.6 mL microcentrifuge tube and lyophilized to dryness using a SpeedVac evaporator. To that lyophilized oligonucleotide mixture was added 5 µL freshly prepared 0.2 M EDC in 0.1 M HEPES, pH 7.5 buffer (pH was adjusted with 0.1 M NaOH solution). The mixture was immediately vortexed, centrifuged and cooled and maintained at 4 °C (or at the appropriate temperature indicated for higher temperature reactions). At different time intervals (as indicated in the Figure) 1 µL of reaction mixture was pipetted out and diluted to 450 µL by adding nuclease free water and immediately frozen in dry-ice to stop the reaction; this sample was analyzed by AEC for monitoring of the progress of the reaction. Most of the experiments were run in triplicate and the error range is less than ± 5%.

General procedure for the step-wise dilution experiment. Properly measured aliquots of template (10 µM) and ligands R_{L3}, R_{L4}, R_{L5}, R_{L6} (20 µM of each) in a microcentrifuge tube at 30 °C was lyophilized to dryness. To the dried mixture, 6 µL of freshly prepared ligation buffer (0.2 M EDC, 0.1 M HEPES, 150 mM NaCl, pH 7.5) was added and the mixture was vortexed followed by centrifugation. After that, 1 µL of the reaction mixture was pipetted off for analysis (see below) and the remaining mixture (5 µL) was allowed to continue at 4 °C. After 24 h, 1 µL of the reaction mixture was again pipet off for analysis. Then, in another microcentrifuge tube, new aliquots of only ligands R_{L3}, R_{L4}, R_{L5}, R_{L6} (20 µM of each and NO TEMPLATE) were lyophilized and 1 µL of freshly prepared ligation buffer was added and the ligands mixture was transferred to the remaining volume (4 µL) of 24 h reaction mixture (final volume became 1+4 = 5 µL). Therefore, the overall template concentration was decreased but the ligands’ concentrations remain the same (20 µM) throughout the reaction. The procedure of removal of 1 µL for analysis followed by addition of fresh ligands was repeated at 48 h and 72 h; at 96 h, only the analysis was performed. Experiments were run in triplicate and the error range is less than ± 5%.

AEC analysis: The 1 µL of the reaction mixture was diluted to 0.5 mL with nuclease free water and 1 µL of 10 µM dT_{24} was added to it (as the external standard), and the mixture was filtered through Millex-GV PVDF filter (4 mm, 0.22µm). Then, the filtered solution was injected to AEC to get the chromatogram trace of the reaction mixture and the remaining reaction mixture was allowed to continue at 4 °C. The same procedure was repeated to get the AEC traces at the 24, 48, 72 and 96 h.
General procedure for T4 DNA Ligase catalyzed template directed enzymatic ligation reaction. In a microcentrifuge tube, appropriate aliquots of template (10 µM) and ligands (10 µM) were lyophilized to dryness at 30 °C in a SpeedVac evaporator. To that dried mixture, 2 µL of T4 DNA ligase buffer (10X), 17 µL of nuclease free water was added and the reaction mixture was heated at 37 °C for 5 min and then cooled at 4 °C. To that cold mixture, 1 µL of 20-unit T4 DNA Ligase (New England Biosciences) was added (final ligase concentration 1 unit/µL) and the reaction was allowed to continue at 4 °C. At different time interval, 1 µL of the reaction mixture was pipet off and stop by heat inactivation at 65 °C for 10 min. Then, 0.5 mL of nuclease free water was added to that aliquot and injected to AEC to monitor the progress of the reaction.

General procedure for T4 RNA Ligase 2 catalyzed template directed enzymatic ligation reaction. In a microcentrifuge tube, appropriate aliquot of template (10 µM) and ligands (10 µM) were lyophilized to dryness at 30 °C in a SpeedVac evaporator. In to that, 2 µL of T4 RNA ligase 2 buffer (10X), 17 µL of nuclease free water was added and the reaction mixture was warmed at 37 °C for 5 min and then cooled at 4 °C for 5 min. To that cold mixture, 1 µL of 10-unit T4 DNA Ligase (from New England Biosciences) was added (final ligase concentration became 0.5 unit/µL) and the reaction was allowed to continue at 4 °C. At different time interval, 1 µL of the reaction mixture was pipet off and stop by heat inactivation at 75 °C for 15 min. Then, 0.5 mL of nuclease free water was added to that aliquot and injected to AEC to monitor the progress of the reaction.

Anion exchange chromatography (AEC) analysis: Analysis was performed by AEC, by monitoring at 260 nm wave length. The instrument, column and the eluting buffer conditions are described in the general part, while the gradient employed for the experiment is mentioned under each AEC trace. In most cases, the signal of the template was used as internal standard for determining the yields of the ligation product(s). The yields were calculated w.r.t. the area of the template peak by using the formulae {((area of ligation product peak/area of template peak) X 100)} at each time point where the area of the peaks was normalized by their respective molar extinction coefficient. The stability of the template in presence of ligation buffer was checked at different time intervals and found to be not degraded when compared to an externally added oligo standard even after 24 h, at 4 °C. In most of the cases, reactions were monitored up to 24 h since inception, and in certain cases it was monitored till 48 h, and for step-wise dilution experiment the reactions were monitored till 96 h. For background reactions (in absence of any template) and in step-wise
dilution experiment the % yields were calculated w.r.t. an externally added standard oligo (dT_{24}) just before the injection in to the AEC. Reactions were run in triplicates and the yield calculation was within ± 5%.

**Buffers for AEC analysis of ligation reaction:** Buffer A: 10 mM Na_{2}HPO_{4}, pH 11. Buffer B: 10 mM Na_{2}HPO_{4}, 1 M NaCl, pH 11. Flow rate: 0.8 mL/min. The gradient profile is specified under each AEC trace.

**Preparation of sample for MALDI-TOF analysis:** The nature of the ligation product was confirmed by the MALDI-TOF analysis of the 24 h reaction mixture. The 24 h reaction mixture was de-salted by using standard ZipTip desalting method.

*ZipTip desalting method:* A ZipTip was attached to a 10 µL micropipette and then washed consecutively with acetonitrile (3 X 4 µL) and 0.1M TEAAc buffer (3 X 4 µL). Then the ZipTip was consecutively pipetted with the reaction mixture (10 times), 0.1M TEAAc (3 times), water (3 times) and finally the reaction mixture is eluted with 2 µL 1:1 acetonitrile/H_{2}O. Then 1 µL of the desalted reaction mixture (in 1:1 MeCN/H_{2}O) was spotted on the MALDI plate with 1 µL of THAP matrix and analyzed.
**Ligation reaction:** TNA ligands ($T_{L1}, T_{L2}$) on an chimeric TRNA template ($C_{T1}$)

![Chemical reaction image](image)

| Entry          | Sequence                        | Expected mass | Observed mass |
|----------------|---------------------------------|---------------|---------------|
| Ligand $T_{L1}$ | 3'-aaaatatat-PO$_3^{2-}$-2'      | 2674          | 2674          |
| Ligand $T_{L2}$ | 3'-OHattatta-2'                 | 1996          | 1996          |
| Template $C_{T1}$ | 3'-TTTTaatAatAAtAAt-3'(5')     | 4923          | 4920          |
| Ligation product $P_{1}$ | 3'-aaaatatatattatta-2' | 4652          | NOT           |
| Yield (%)       | 1 h 24 h 48 h                   | 0% 7% 13%     | Observed      |

**Supplementary Figure 14.** AEC trace of the ligation reaction of TNA ligands (0.2 mM of each) on TNA-RNA chimeric template (0.1 mM) in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate the appearance of ligation product peak at lower retention time than the template peak (Top trace). The rate of reaction is very slow even MALDI spectrum of the 24 h reaction mixture could not detect the ligation product formation (Bottom table) which was further confirmed by comparing with the standard ligation product. *Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.*
**Ligation reaction**: TNA ligands (T\textsubscript{L1}, T\textsubscript{L2}) on an RNA template (R\textsubscript{T1})

\[ 3'\text{-}\text{aa a a t t a t -PO}_3^{2\cdot} + ^{\text{HO}}\text{a t t a t a -2'} \rightarrow 3'\text{-}\text{U U U U A A U A U A U A U A U A U -5'} \]

**Supplementary Figure 15.** AEC trace of the ligation reaction of TNA with 2'-OH ligands (0.2 mM of each) on RNA template (0.1 mM) in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate the appearance of ligation product peak at lower retention time than the template peak. The rate of reaction is very slow compared to the homogeneous TNA ligands with 2'-NH\textsubscript{2}. The ligation product peak was observed only after 24 h of reaction (Top trace). MALDI spectrum of the 24 h reaction mixture confirms the ligation product formation (Bottom table). *Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.*
Ligation reaction: RNA ligands (5'-PO$_3^{2-}$ and 3'-OH) on RNA template (R$_{T1}$)

5'-AAA AUUA$^\text{OH}$ + PO$_3^{2-}$-UAUUUAUUA-3'  3'-UUUUAAAU  AUAUAAAU-5'  

0.2M EDC, 0.1M HEPES, pH 7.5, 4 °C

Supplementary Figure 16. AEC trace of the ligation reaction of canonical RNA ligands (0.2 mM of each) on RNA template (0.1 mM) in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate that even after 24 h of reaction no ligation product peak appeared which was confirmed by spiking with authentic product (0.14 mM). The AEC traces indicate the difference in retention time between the template and the ligation product. **Gradient:** 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
**Ligation reaction:** TRNA ligands (5'-PO$_3^{2-}$ and 2'-OH) on RNA template (R$_{T1}$)

(5')3'-aa a aT TTA t$^{OH}$ PO$_3^{2-}$-A t t A t t A-3'
3'-UUUUAAUA----- UAAUAU-5'

0.2M EDC, 0.1M HEPES, pH 7.5, 4 °C

Supplementary Figure 17. AEC trace of the ligation reaction of TRNA chimeric ligands (5'-PO$_3^{2-}$ and 2'-OH) (0.2 mM of each) on RNA template (0.1 mM) in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate that even after 24 h of reaction a minor ligated product peak appeared which was confirmed by spiking with authentic product (0.15 mM). The AEC trace indicate the difference in retention time between the template and the ligation product. The broad peaks with minor shoulders are due to the background from column. Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
**Ligation reaction:** TRNA chimeric ligands (C₁₁,C₁₂) on chimeric TRNA template (Cₜ₁)

\[
\begin{align*}
(5')3'\text{-}aaa\text{TTTAA}^{\text{NH}}\text{TPO}_3^2\text{At tA}-3' \\
3'\text{-}TTTTaa\text{tA} & \quad t\text{AAAAt-3'}(5') \\
& \quad \rightarrow \\
(5')3'\text{-}aaa\text{TTTAA(NH)} \text{At tA}-3' \\
3'\text{-}TTTTaa\text{tA} & \quad t\text{AAAAt-3'}(5')
\end{align*}
\]

**Supplementary Figure 18.** AEC trace of ligation reaction of TNA-RNA chimeric ligands (0.2 mM of each) in presence of TNA-RNA chimeric template (0.1 mM) in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate that even after 24 h of reaction no peak corresponds to the ligation product is detected (Top trace) although the MALDI spectrum of the 24 h reaction mixture confirms the ligation product peak (Bottom table). Gradient: 0 to 40% Buffer B in 5 min then 40 to 90% Buffer B in 30 min.

| Entry          | Sequence                        | Expected mass | Observed mass |
|----------------|---------------------------------|---------------|---------------|
| Ligand C₁₁    | (5')3'-aaa\text{TTTAA}^{\text{NH}}\text{TPO}_3^2\text{At tA}-3' | 2727          | 2729          |
| Ligand C₁₂    | 5'-PO₃²⁻-\text{AttAtA-3'}       | 2166          | 2168          |
| Template Cₜ₁  | 3'-TTTTaa\text{tAtAAtAAt-3'}(5') | 4923          | 4925          |
| Ligation product Cₚ₁ | (5')3'-aaa\text{TTTAA(NH)AttAtA-3'} | 4876          | 4876          |

(Not observed in AEC)
**Ligation reaction:** RNA ligands (R_{L1}, R_{L2}) on chimeric TRNA template (C_{T1})

\[
\begin{align*}
5'-\text{AAAATTTA}_\text{NH}_2\text{T} & \quad \text{PO}_3^2\text{-ATTATTA-3'} \\
3'-\text{TTTTaaat tA} & \quad \text{IAAAtA-3'(5')}
\end{align*}
\]

\[
\text{0.2M EDC, 0.1M HEPES, pH 7.5 buffer.}
\]

Supplementary Figure 19. AEC trace of the ligation reaction of RNA ligands (0.2 mM of each) on TNA-RNA chimeric template (0.1 mM) in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate that appearance of ligation product peak at higher retention time than the template peak (Top trace). MALDI of the 24 h reaction mixture confirms the ligation reaction (Bottom table). * unassigned background peak.

| Entry          | Sequence                                           | Expected mass | Observed mass |
|----------------|----------------------------------------------------|---------------|---------------|
| Ligand R_{L1}  | 5'-AAAATTTAT_{NH2} -3'                             | 2847          | 2852          |
| Ligand R_{L2}  | 5'-PO_3^2-ATTATTA-3'                               | 2286          | 2289          |
| Template C_{T1}| 3'-TTTTaatAtAAatAt-3'(5')                         | 4923          | 4928          |
| Ligation product R_{P1} | 5'-AAAATTTAT_{NH}ATTATTA-3' | 5116          | 5122          |
| Yield (%)      | 1 h 3 h 24 h                                       | 23% 51% 102%  |               |

*Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.*
**Ligation reaction:** TNA ligands (T₇₁, T₇₃) on chimeric RTNA template (C₇₁)

\[
3'\text{-}\text{aaaa} \ t \ t \ t \ a \ t \text{-PO₃}^{2-} \ \text{NH₂} \text{-}a \ t \ t \ t \ t \ a \text{-}2'
\]
\[
3'\text{-TTT} \text{aaa} \ t \text{A} + \text{tAA} \text{AAt-3'(5')} \rightarrow 0.2 \text{M EDC, 0.1 M HEPES, pH 7.5}
\]
\[
3'\text{-a a a} \ a \ t \ t \ t \ a \ t \ (\text{NH}) \text{-}a \ t \ t \ t \ t \ a \text{-}2'
\]

**Supplementary Figure 20.** AEC traces of the ligation reaction of homogeneous TNA ligands (0.2 mM of each) on TNA-RNA chimeric template (0.1 mM) in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate the appearance of ligation product peak at lower retention time than the template peak (Top trace). MALDI of the 24 h reaction mixture confirms the ligation reaction (Bottom table). *Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.*
**Ligation reaction:** TRNA chimeric ligands (C₁₈,C₁₂) on RNA template (R₁₈)

\[
\begin{align*}
3'-\text{aaaa TTT} & \overset{\text{NH₂}}{\text{TTA}} - \overset{\text{PO₃²⁻}}{\text{AttA}} - 3' \\
3'-\text{UUUAAUAUA} & + \text{UAUAAAU-5'} \\
\end{align*}
\]

\[0.2 \text{M EDC, 0.1 M HEPES, pH 7.5 buffer.}\]

AEC trace of the ligation reaction of TNA-RNA chimeric ligands (0.2 mM of each) on RNA template (0.1 mM) on 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate the appearance of ligation product peak at lower retention time than the template peak (Top trace). MALDI of the 24 h reaction mixture confirms the ligation reaction (Bottom table).

**Supplementary Figure 21.** AEC trace of the ligation reaction of TNA-RNA chimeric ligands (0.2 mM of each) on RNA template (0.1 mM) on 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate the appearance of ligation product peak at lower retention time than the template peak (Top trace). MALDI of the 24 h reaction mixture confirms the ligation reaction (Bottom table). *Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.* * indicates the unassigned peaks.
**Ligation reaction**: RNA ligands (R\textsubscript{L1}, R\textsubscript{L2}) on RNA template (R\textsubscript{T1})

\[
\begin{align*}
3'\text{-}AAATTT\text{A}^\text{NH}_2 &\quad \text{PO}_3^2\text{-}\text{ATTATTA}-3' \\
3'\text{-}UUUAAUA &\quad \text{UAUAAU-5'} \\
\xrightarrow{0.2\text{M EDC, } 0.1\text{M HEPES, pH 7.5 buffer}} &\quad (5')3'\text{-}AAATTT\text{A}(\text{NH})\text{ATTATTA}-3' \\
&\quad 3'\text{-}UUUAAUA &\quad \text{UAUAAU-5'}
\end{align*}
\]

**Supplementary Figure 22.** AEC trace of the ligation reaction of RNA ligands (0.2 mM of each) on RNA template (0.1 mM) on 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate the appearance of ligation product peak at lower retention time than the template peak (Top trace). MALDI of the 24 h reaction mixture confirms the ligation reaction (Bottom table). Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.

| Entry                  | Sequence                        | Expected mass | Observed mass |
|------------------------|---------------------------------|---------------|---------------|
| Ligand R\textsubscript{L1} | 5'\text{-}AAAATTTA^\text{NH}_2-3' | 2847          | 2850          |
| Ligand R\textsubscript{L2} | 5'\text{-}PO_3^2\text{-}ATTATTA-3' | 2286          | 2287          |
| Template R\textsubscript{T1} | 3'\text{-}UUUAAUAUAUAUAUAU-5' | 5021          | 5023          |
| Ligation product R\textsubscript{P1} | 5'\text{-}AAAATTTA(NH)ATTATTA-3' | 5116          | 5118          |

Yield (%)

| Time (h) | 1 h | 3 h | 24 h |
|----------|-----|-----|------|
| Yield (%)| 43% | 81% | 102% |
**Ligation reaction:** TNA ligands (T_{L1}, T_{L3}) on RNA template (R_{T1})

Supplementary Figure 23. AEC trace of the ligation reaction of homogeneous TNA ligands on homogeneous RNA template in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer (Top trace). The AEC traces indicate the appearance of ligation product peak at lower retention time than the template peak. MALDI of the 24 h reaction mixture confirms the ligation reaction (Bottom table). **Gradient:** 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
**Ligation reaction:** Competition among TRNA (C\textsubscript{L1},C\textsubscript{L2}) and RNA ligands (R\textsubscript{L1},R\textsubscript{L2}) on chimeric TRNA template (C\textsubscript{T1})

\[ 5'-\text{AAAATTATTA}\textsuperscript{NH2}-3' + 5'-\text{PO}_3\textsuperscript{2-}ATATTATA-3' \]
\[ + + \]
\[ (5')3'-aaaATTTATTA\textsuperscript{NH2}-3' + 5'-\text{PO}_3\textsuperscript{2-}ATTAtA-3' \]

\[ 3'-\text{TAAAAAtAAtAat-3'(5')} \]

**Supplementary Figure 24.** AEC traces of the competitive ligation reaction of RNA (R\textsubscript{L1},R\textsubscript{L2}) and chimeric TRNA (C\textsubscript{L1},C\textsubscript{L2}) ligands (0.2 mM of each) on TRNA-chimeric template (C\textsubscript{T1}, 0.1 mM) in 0.2 M EDC, 0.1 M HEPES, pH 7.5 buffer. The AEC traces indicate the selection of only homogeneous ligands from the mixture (Top trace). MALDI of the 24 h reaction mixture confirms the ligation reaction (Bottom table). **Gradient:** 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.

| Entry | Sequence | Expected mass | Observed mass |
|-------|----------|---------------|---------------|
| Ligand R\textsubscript{L1} | 5'-AAAATTATTA\textsuperscript{NH2}-3' | 2847 | 2846 |
| Ligand R\textsubscript{L2} | 5'-\text{PO}_3\textsuperscript{2-}ATATTATA-3' | 2286 | 2284 |
| Ligand C\textsubscript{L1} | (5')3'-aaaATTTATTA\textsuperscript{NH2}-3' | 2727 | 2726 |
| Ligand C\textsubscript{L2} | 5'-\text{PO}_3\textsuperscript{2-}ATTAtA-3' | 2166 | 2165 |
| Template C\textsubscript{T1} | 3'-TAAAAAtAAtAat-3'(5') | 4923 | 4920 |
| Homogeneous Ligation product R\textsubscript{P1} | 5'-AAAATTATTA\textsuperscript{(NH)}ATATTATA-3' | 5116 | 5113 |
| | 1 h | 4 h | 24 h |
| | 47% | 86% | 107% |
| Heterogeneous Ligation product C\textsubscript{P1} | (5')3'-aaaATTTATTA\textsuperscript{(NH)}AttAtA-3' | 4876 | Not Observed |
**Control reaction:** TRNA chimeric ligands (C_{L1}, C_{L2}) in absence of any template

![AEC traces of the background ligation reaction of TNA-RNA chimeric ligands (0.2 mM of each) in absence of any template in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate that even after 24 h of reaction no peak corresponds to the ligation product is detected (Top trace). MALDI of the 24 h reaction mixture. It confirms the absence of the ligation product peak (Bottom table). **Gradient:** 0 to 40% Buffer B in 5 min then 40 to 90% Buffer B in 25 min.]

| Entry          | Sequence                        | Expected mass | Observed mass |
|----------------|---------------------------------|---------------|---------------|
| Ligand C_{L1}  | (5')3'-aaaaTTTAT^{NH2}          | 2727          | 2728          |
| Ligand C_{L2}  | (5')-PO_3^2-AttAttA-3'          | 2166          | 2166          |
| Ligation       | product C_{P1} (5')3'-aaaaTTTAT(NH)AttAttA-3' | 4876          | NOT Observed  |

**Supplementary Figure 25.** AEC traces of the background ligation reaction of TNA-RNA chimeric ligands (0.2 mM of each) in absence of any template in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate that even after 24 h of reaction no peak corresponds to the ligation product is detected (Top trace). MALDI of the 24 h reaction mixture. It confirms the absence of the ligation product peak (Bottom table). **Gradient:** 0 to 40% Buffer B in 5 min then 40 to 90% Buffer B in 25 min.
**Control reaction:** RNA ligands (R_{L1}, R_{L2}) in absence of any template

\[ \text{0.2 M EDC, 0.1 M HEPES, pH 7.5, 4 °C} \]

\[ 5'\text{-AAAATTTA}_T^{NH2}\text{-3'} + 5'\text{-PO}_3^{2-}\text{ATTATTA-3'} \]

**Supplementary Figure 26.** AEC trace of the background ligation reaction of homogeneous RNA ligands (0.2 mM of each) in absence of any template in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate that even after 24 h of reaction no peak corresponds to the ligation product is detected (Top trace). MALDI of the 24 h reaction mixture also confirms the absence of the ligation product peak (Bottom table). **Gradient:** 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
Control reaction: TNA ligands (T_{L1}, T_{L3}) in absence of any template

0.2M EDC, 0.1M HEPES, pH 7.5, 4 °C

3'-aaaattt-PO_{3}^{2-} -\text{NH}^{2}attatta-2'

Supplementary Figure 27. AEC trace of the background ligation reaction of TNA ligands (0.2 mM of each) in absence of any template in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate that even after 24 h of reaction no peak corresponds to the ligation product is detected (Top trace). (Bottom table) MALDI of the 24 h reaction mixture also confirms the absence of the ligation product peak. Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
**Control reaction**: TNA ligands (T_{L1}, T_{L2}) in absence of any template.

```
0.2M EDC, 0.1M HEPES, pH 7.5, 4 °C
```

\[
3'\text{-aaaattat-PO}_3^{2-} + \text{OH}\text{attatta-2'} \rightarrow \text{No ligation product}
\]

![Absorbance trace at 260 nm](image)

| Entry          | Sequence                                      | Expected mass | Observed mass |
|----------------|-----------------------------------------------|---------------|---------------|
| Ligand T_{L1}  | 3'\text{-aaaattat-PO}_3^{2-} -2'              | 2674          | 2676          |
| Ligand T_{L2}  | 3'\text{-OH}\text{attatta-2'}               | 1994          | 1997          |
| Ligation product T_{P1} | 3'\text{-aaaattatattatta-2'} | 4652          | NOT Observed  |

**Supplementary Figure 28.** AEC trace of the background ligation reaction of TNA ligands with 2'-OH (0.2 mM of each) in absence of any template in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate that even after 24 h of reaction no peak corresponds to the ligation product is detected (Top trace). MALDI of the 24 h reaction mixture also confirms the absence of the ligation product peak (Bottom table). Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
### Supplementary Table 4: AEC and MALDI-TOF data for DNA-RNA chimeric oligonucleotide sequences synthesized.

| Entry | Sequence | retention time [min] | MALDI-TOF-MS (Calculated) [M + H]^+ | MALDI-TOF-MS (Observed)* |
|-------|----------|----------------------|--------------------------------------|---------------------------|
| 1     | 5'-UUUUAAAT\textsuperscript{NH2}-3' | 10.1\textsuperscript{a} | 2405                                 | 2406                      |
| 2     | 5'-UUUUAATA\textsuperscript{NH2}-3' | 10.1\textsuperscript{a} | 2453                                 | 2454                      |
| 3     | 5'-TTTTAAAT\textsuperscript{NH2}-3' | 9.5\textsuperscript{a} | 2397                                 | 2396                      |
| 4     | 5'-UUUAUU\textsuperscript{NH2}-3' | 10.2\textsuperscript{a} | 2453                                 | 2453                      |
| 5     | 5'-GUC\textsuperscript{NH2}-3' | 14.5\textsuperscript{b} | 1197                                 | 1198                      |
| 6     | 5'-GTC\textsuperscript{NH2}-3' | 14.3\textsuperscript{b} | 1195                                 | 1197                      |
| 7     | 5'-UGC\textsuperscript{NH2}-3' | 14.4\textsuperscript{b} | 1197                                 | 1199                      |
| 8     | 5'-PO_3\textsuperscript{2-}-ACGA-3' | 14.4\textsuperscript{b} | 1326                                 | 1327                      |
| 9     | 5'-PO_3\textsuperscript{2-}-ACG\textsuperscript{4}-3' | 14.4\textsuperscript{b} | 1294                                 | 1295                      |
| 10    | 5'-PO_3\textsuperscript{2-}-AGAC-3' | 14.3\textsuperscript{b} | 1326                                 | 1327                      |
| 11    | 5'-UUUUAAAT(NH)AUUAAU-3' | 20.6\textsuperscript{c} | 4890                                 | 4886                      |
| 12    | 5'-UUUAAGAAT(NH)AUAAUA-3' | 22.2\textsuperscript{c} | 5018                                 | 5015                      |
| 13    | 5'-TTTTAAAT(NH)AUAAUA-3' | 25.5\textsuperscript{c} | 4962                                 | 4960                      |

Oligonucleotides 1-10 were synthesized by using Expedite synthesizer and oligonucleotides 11-13 were synthesized by EDC-mediated non-enzymatic ligation reactions, and purified by AEC; \( ^a \) 0 to 100% buffer B gradient using 5 mM Tris base, pH 8.2 as Buffer A and 5 mM Tris base, 330 mM NaClO\textsubscript{4}, pH 8.2 as Buffer B; \( ^b \) 0 to 100% buffer B gradient using 10 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 11 as Buffer A and 10 mM Na\textsubscript{2}HPO\textsubscript{4}, 1 M NaCl, pH 11 as Buffer B; \( ^c \) 0 to 40% buffer B in 1 min then 40 to 90% buffer B in 30 min gradient using 10 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 11 as Buffer A and 10 mM Na\textsubscript{2}HPO\textsubscript{4}, 1 M NaCl, pH 11 as Buffer B; ; peak purity (260 nm) \( \geq 95\% \); desalting on Sep-Pak cartridges. \( ^{S2} \) MALDI-TOF spectrum was recorded using THAP matrix; A,U, G,C = RNA; A,T,C = DNA.
**Supplementary Table 5**: Thermal stability of duplexes (c = 2+2 µM) formed from RDNA chimeric sequences and the corresponding parent DNA and RNA sequences.

| Entry | Duplex | Description | UV-Tm (°C)\(^b\) | ΔTm (°C)\(^#\,b\) |
|-------|--------|-------------|-----------------|-----------------|
| AU, \(AT\) and parent RNA sequences | | | | |
| 1. | 5\(^\prime\)-AAAAUUUATA\(\text{TTTAA}T\)-3\(^\prime\) + 3\(^\prime\)-UUUU\(\text{AA}T\)A\(\text{AA}T\)-5\(^\prime\) | RDNA | 32.8\(^a\) | -12.6 |
| | | RDNA | 23.6 | |
| 2. | 5\(^\prime\)-AAAAUUUATA\(\text{TAT}T\) A-3\(^\prime\) + 3\(^\prime\)-UUUU\(\text{AA}A\)AUUUA\(\text{A}A\)UU-5\(^\prime\) | RNA | 35.2\(^a\) | -9.9 |
| | | RDNA | 26.5 | |
| 3. | 5\(^\prime\)-AAA\(\text{UU}U\)AAUUAUUA-3\(^\prime\) + 3\(^\prime\)-UUUU\(\text{AA}A\)TT\(\text{AA}T\)AT\(\text{AA}T\)-5\(^\prime\) | RNA | 36.3\(^a\) | -24.8 |
| | | RDNA | 19.9\(^a\) | <5\(^d\) |
| 4. | 5\(^\prime\)-UUUU\(\text{AA}A\)UU\(\text{AA}A\)AUU-3\(^\prime\) + 3\(^\prime\)-AAAAUUUA\(\text{U}U\)UU\(\text{U}U\)-5\(^\prime\) | RDNA | 20.1\(^b,c\) | -14.5 |
| | | RNA | 27.2\(^b,c\) | -12.9 |
| 5. | 5\(^\prime\)-UUUU\(\text{AA}A\)AT\(\text{NH}\)\(\text{U}U\)\(\text{AA}A\)AAUU-3\(^\prime\) + 3\(^\prime\)-AAAAUUUA\(\text{U}U\)UU\(\text{U}U\)-5\(^\prime\) | RDNA\((\text{NH})\) | 11.6 | -23.8 |
| | | RNA | 27.9 | -7.5 |
| 6. | 5\(^\prime\)-UUUU\(\text{AA}A\)UAAUUUA\(\text{A}A\)UU-3\(^\prime\) + 3\(^\prime\)-AAAAUUUA\(\text{A}A\)UU\(\text{U}U\)-5\(^\prime\) | RDNA\((\text{NH})\) | 20.1\(^b,c\) | -14.0 |
| | | RNA | 27.2\(^b,c\) | -12.9 |
| 7. | 5\(^\prime\)-UUUU\(\text{AA}A\)AT\(\text{NH}\)U\(\text{A}A\)U\(\text{A}A\)UAUU-3\(^\prime\) + 3\(^\prime\)-AAAAUUUA\(\text{U}U\)UU\(\text{U}U\)-5\(^\prime\) | RDNA\((\text{NH})\) | 11.6 | -23.8 |
| | | RNA | 27.9 | -7.5 |
| 8. | 5\(^\prime\)-UUUU\(\text{AA}A\)AA\(\text{A}A\)U\(\text{A}A\)AAU-3\(^\prime\) + 3\(^\prime\)-AAAA\(\text{U}U\)UAA\(\text{U}U\)AUUA-5\(^\prime\) | RNA | 20.1\(^b,c\) | -14.3 |
| | | RDNA \((\text{NH})\) | 21.5 | -13.9 |
| 9. | 5\(^\prime\)-UUUU\(\text{AA}A\)AT\(\text{NH}\)\(\text{U}U\)\(\text{AA}A\)AAUU-3\(^\prime\) + 3\(^\prime\)-AAAA\(\text{U}U\)UAA\(\text{U}U\)UUAUA-5\(^\prime\) | RNA | 15.8\(^b,c\) | -19.6 |
| | | DNA | 19\(^a\) | -5\(^d\) |
| 10. | 5\(^\prime\)-\(\text{T}T\)\(\text{T}A\)\(\text{AA}T\)\(\text{A}A\)\(\text{A}A\)\(\text{T}\)-3\(^\prime\) + 3\(^\prime\)-UUUU\(\text{AA}A\)UU\(\text{U}U\)UU\(\text{U}U\)-5\(^\prime\) | DNA | 19\(^a\) | -5\(^d\) |
| | | DNA | <5\(^d\) | |
| 11. | 5\(^\prime\)-UUUU\(\text{AA}A\)AT\(\text{A}A\)U\(\text{U}U\)\(\text{A}A\)UU-3\(^\prime\) + 3\(^\prime\)-AAAA\(\text{U}U\)UU\(\text{U}U\)UU\(\text{U}U\)-5\(^\prime\) | RDNA | 35\(^a\) | -14.9 \(^d\) |
| Entry | Duplex | Description | UV-Tm (°C)\(^b\) | ΔT\(_m\) (°C)\(^b,c\) |
|-------|--------|-------------|------------------|-----------------|
| **AUGC, ATGC sequences and parent RNA sequences** | | | | |
| 13. | 5'-UUUU<sub>4</sub>GU<sub>4</sub>CA<sub>4</sub>GUAU<sub>4</sub>U-3' + 3'-<sub>4</sub>A<sub>4</sub>U<sub>4</sub>U<sub>4</sub>U<sub>4</sub>GUC<sub>4</sub>U<sub>4</sub><sub>4</sub>A<sub>4</sub>U<sub>4</sub>U<sub>4</sub>U<sub>4</sub>U<sub>4</sub>-5' | RDNA | 26.7 | -24.6 |
| 14. | 5'-UUUU<sub>A</sub>AGU<sub>A</sub>CAGUA<sub>A</sub>A <sub>U</sub>-3' + 3'-<sub>A</sub>A<sub>A</sub>U<sub>A</sub>U<sub>A</sub>U<sub>A</sub>GUC<sub>U</sub><sub>A</sub>U<sub>A</sub>A<sub>U</sub><sub>A</sub>-5' | RDNA | 36.9 | -14.4 |
| 15. | 5'- UUUA<sub>A</sub>AGU<sub>A</sub>CAGUA<sub>A</sub>AU<sub>A</sub>-3' + 3'-<sub>A</sub>A<sub>A</sub>A<sub>A</sub>U<sub>A</sub>U<sub>A</sub>U<sub>A</sub>U<sub>A</sub>GUC<sub>A</sub>U<sub>A</sub>A<sub>U</sub><sub>A</sub>-5' | RNA | 44.1 | -7.2 |
| 16. | 5'-G<sub>T</sub>T<sub>C</sub>T<sub>A</sub>C<sub>G</sub>A<sub>4</sub>-3' + 3'-<sub>C</sub>AGAT<sub>G</sub>G<sub>C</sub>U<sub>4</sub>-5' | RDNA | 18.6 | -21.5 |
| 17. | 5'- G<sub>T</sub>T<sub>C</sub>T<sub>A</sub>C<sub>G</sub>A<sub>4</sub>-3' + 3'-<sub>C</sub>AGA<sub>G</sub>U<sub>C</sub>G<sub>U</sub>-5' | RNA | 31.8 | -8.3 |
| 18. | 5'-GU<sub>C</sub>U<sub>A</sub>C<sub>G</sub>A<sub>4</sub>-3' + 3'-<sub>C</sub>AGAT<sub>G</sub>G<sub>C</sub>U<sub>4</sub>-5' | RNA | 27.6 | -12.5 |
| **RNA series for comparison** | | | | |
| 19. | 5'-<sub>A</sub>A<sub>A</sub>A<sub>A</sub>U<sub>A</sub>A<sub>A</sub>A<sub>A</sub>A<sub>A</sub>A<sub>A</sub>-3' + 3'-<sub>A</sub>A<sub>A</sub>A<sub>A</sub>U<sub>A</sub>A<sub>A</sub>A<sub>A</sub>A<sub>A</sub>-5' | RNA | 36.4\(^b,c\) | |
| 20. | 5'-<sub>A</sub>A<sub>A</sub>A<sub>A</sub>U<sub>A</sub>A<sub>A</sub>A<sub>A</sub>A<sub>A</sub>A<sub>A</sub>-3' + 3'-<sub>A</sub>A<sub>A</sub>A<sub>A</sub>U<sub>A</sub>A<sub>A</sub>A<sub>A</sub>A<sub>A</sub>-5' | RNA | 35.4\(^b,c\) | |
| 21. | 5'-<sub>A</sub>A<sub>A</sub>A<sub>A</sub>U<sub>A</sub>A<sub>A</sub>A<sub>A</sub>A<sub>A</sub>A<sub>A</sub>-3' + 3'-<sub>A</sub>A<sub>A</sub>A<sub>A</sub>U<sub>A</sub>A<sub>A</sub>A<sub>A</sub>A<sub>A</sub>-5' | RNA | 51.3 | |
| 22. | 5'-GU<sub>C</sub>U<sub>A</sub>C<sub>G</sub>A<sub>4</sub>-3' + 3'-<sub>C</sub>AGA<sub>G</sub>U<sub>C</sub>G<sub>U</sub>-5' | RNA | 40.1 | |

Duplex concentration = 2±2 µM; \(a\) = 1M NaCl, 10 mM Na2HPO4, 100 µM EDTA pH 7.2 buffer; \(b\) = 150 mM NaCl, 10 mM Na2HPO4, 100 µM EDTA pH 7.2 buffer; \(c\) = Reference 5; \(d\) = 0.1M HEPES, pH 7.5. \# ΔT\(_m\) is with respect to the corresponding parent RNA duplex. A, U, G, C = RNA; A, T, G, C = DNA.
UV melting curves

Supplementary Figure 29. Comparison of UV-\(T_m\) curves of duplexes formed by non-self-complementary RDNA chimeric strand with its complementary RNA sequence versus the duplex formed with its complementary RDNA chimeric strand (Supplementary Table 5, Entry 1-3) in 10 mM Na\(_2\)HPO\(_4\), and 150 mM NaCl, 100 \(\mu\)M EDTA at pH 7.2 buffer. A, U = RNA; \(\text{T, T'} = \text{DNA.}\)
Supplementary Figure 30. Effect of salt concentration on UV-\(T_m\) curves of duplexes formed by non-self-complementary RDNA chimeric strand in 10 mM Na\(_2\)HPO\(_4\), 100 µM EDTA, pH 7.2 (150 mM and 1M NaCl, Supplementary Table 5, Entry 4, 6 buffers \(a, b\) respectively) and in 0.1 M HEPES at pH 7.5 (no salt, Supplementary Table 5, Entry 4,6, buffer \(d\)). A, U = RNA; \(\text{A} = \text{DNA}\).
Supplementary Figure 31. Comparison of UV-Tm curves of duplexes formed from RDNA chimeric sequences with phosphodiester linkage (blue curve) and with corresponding T phosphoramidate linkage (red curve), demonstrating similar thermal stability. (Supplementary Table 5, Entry 4-5) in 10 mM Na$_2$HPO$_4$, and 150 mM NaCl, 100 µM EDTA at pH 7.2 buffer.
Supplementary Figure 32. Comparison of UV-Tm curves of duplexes formed from RDNA chimeric sequences and its complementary homogeneous RNA strand, with phosphodiester linkage (blue) and with corresponding T phosphoramidate linkage (red) demonstrating similar thermal stability. (Supplementary Table 5, Entry 6-7) in 10 mM Na$_2$HPO$_4$, and 150 mM NaCl, 100 µM EDTA at pH 7.2 buffer.
Supplementary Figure 33. Effect of salt concentration on UV-Tm curves of non-self-complementary RDNA chimeric strands with its complementary chimeric and RNA strand with single T in 10 mM Na₂HPO₄, and 1 M NaCl, 100 µM EDTA at pH 7.2 (Supplementary Table 5, Entry 1-12, buffer a) versus 0.1 M HEPES at pH 7.5 (Supplementary Table 5, Entry 11-12, buffer d). A, U = RNA; A, T = DNA.
**Supplementary Figure 34.** Comparison of UV-$T_m$ curves of non-self-complementary RDNA chimeric strands with its complementary homogeneous RNA and chimeric strands containing all four nucleobases (Supplementary Table 5, Entry 13-15,21) in 10 mM Na$_2$HPO$_4$, and 150 mM NaCl, 100 µM EDTA at pH 7.2 buffer. A, U, G, C = RNA; $\Delta$, $\Lambda$ = DNA.
Supplementary Figure 35. Comparison of UV-$T_m$ curves of shorter (octameric) non-self-complementary RDNA chimeric strands with its complementary homogeneous RNA and chimeric strands containing all four nucleobases (Supplementary Table 5, Entry 16-18,22) in 10 mM Na$_2$HPO$_4$, and 150 mM NaCl, 100 µM EDTA at pH 7.2 buffer. A, U, G, C = RNA; $A$, $T$, $G$, $C$ = DNA.
**Ligation reaction**: RDNA chimeric ligands on a RDNA chimeric template

\[
5'-UUUUAAAT^{NH2}-3' + 5'-PO_3^{2-}-AUAAUAU-3'
\]

\[
3'-AAAAUUUU - AAAUUUU-5'
\]

0.2 M EDC, 0.1 M HEPES, pH 7.5, 4°C

**Supplementary Figure 36.** AEC traces of the ligation reaction of heterogeneous RDNA ligands (0.2 mM of each) on heterogeneous RDNA template (0.1 mM) with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The peak corresponding to the ligation product appeared at lower retention time compared to the template peak (Top trace). In MALDI (Bottom table) of the 24 h reaction mixture the ligation product peak is assumed to be hidden under the template peak which was further confirmed by recording MALDI of the isolated peak.

**Gradient**: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
**Ligation reaction**: RNA ligands on a RDNA chimeric template

\[
5'-UUUAAA{\text{A}}_{NH2}^3' + 5'-PO_3^{2-}-AUAAUAU-3' \\
3'-'\text{AAAAUUAUU} \quad \text{UAUUAAU}^5'
\]

\[0.2 \text{ M EDC, 0.1 M HEPES, pH7.5 , 4°C}\]

| Entry          | Sequence                     | Expected mass | Observed mass |
|----------------|------------------------------|---------------|---------------|
| Ligand R_L3   | 5'-UUUAAA{\text{A}}_{NH2}    | 2453          | 2454          |
| Ligand R_L4   | 5'-PO_3^{2-}-AUAAUAU-3'      | 2582          | 2583          |
| Template C_T2 | 3'-\text{AAAAUUUU}UAUUUUUU-5' | 4893          | 4893          |
| Ligation      | 5'-UUUAAA{\text{A}}_{(NH)}AUAAUAU-3' | 5018          | 5018          |
| Yield (%)      | 1 h 4 h 24 h                 | 57% 91% 103%  |

**Supplementary Figure 37.** AEC traces of the ligation reaction of RNA ligands (0.2 mM each) on heterogeneous RDNA template (0.1 mM) with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The peak corresponding to the ligation product appeared in lower retention time than the template peak (Top trace). MALDI of the 24 h reaction mixture also confirmed the presence of the ligation product peak (Bottom table). Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
**Ligation reaction**: DNA ligands on a RDNA chimeric template

\[ 5'\text{-TTTTAAAT}^{\text{NH2}}\text{-3'} + 5'\text{-PO}_3^{2-}\text{-ATAATAAT}\text{-3'} \rightarrow \text{Ligation product} \]

0.2 M EDC, 0.1 M HEPES, pH 7.5, 4°C

Supplementary Figure 38. AEC traces of the ligation reaction of homogeneous DNA ligands (0.2 mM of each) on heterogeneous RDNA template (0.1 mM) with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The peak corresponding to the ligation product appeared at lower retention time than the template peak (Top trace). MALDI of the 24 h reaction mixture also confirmed the presence of the ligation product peak (Bottom table). *Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.*
**Ligation reaction:** RDNA chimeric ligands on an RNA template

\[
5'-UUUUAAAT^{NH2}-3' + 5'-PO_3^{2-}AUAAUAUU-3' \\
3'-AAAAUUUA \quad \text{UAUUAUU-5'}
\]

0.2 M EDC, 0.1 M HEPES, pH7.5, 4°C

---

**Supplementary Figure 39.** AEC traces of the ligation reaction of RDNA chimeric ligands (0.2 mM each) on an RNA template (0.1 mM) with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The peak corresponding to the ligation product appeared at lower retention time than the template peak (Top trace). MALDI mass analysis of the 24 h reaction mixture also confirmed the presence of the ligation product peak (Bottom table). Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
**Ligation reaction:** RNA ligands on an RNA template

\[
\begin{align*}
5'\text{-UUUAAA}_5^{\text{NH}_2} \quad 5'\text{-PO}_3^-\text{-AUAUAAU}_3' \\
3'\text{-AAAAUUUA} \quad \text{UAUUAUUA}_5'
\end{align*}
\]

**Supplementary Figure 40.** AEC traces of the ligation reaction of homogeneous RNA ligands (0.2 mM) of each on homogeneous RNA template (0.1 mM) in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer with template and ligands. The peak corresponding to the ligation product appeared at lower retention time than the template peak (Top trace). In MALDI of the 24 h reaction mixture the template peak is hidden under the ligation product peak (Bottom table). Ligation product peak was isolated and further confirmed by MALDI-TOF.

Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
**Ligation reaction**: RNA ligands on a RDNA chimeric template

\[ 5'-UUUUAAAU \rightarrow AUAAUAU-3' \]
\[ 3'-AAAUAUUUAPO_3^{-2}\rightarrow \text{NH}_2TAUUAUUA-5' \]

\[ 0.2 \text{ M EDC, 0.1 M HEPES, pH 7.5, 4}^\circ\text{C} \]

Supplementary Figure 41. AEC traces of the ligation reaction of RNA ligands (0.2 mM of each) on RDNA chimeric template (0.1 mM) with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer with template and ligands. The peak corresponding to the ligation product appeared at higher retention time than the template peak (Top trace). MALDI of the 24 h reaction mixture also confirmed the presence of the ligation product peak (Bottom table). Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min. Peaks marked with * refer to unassigned peaks.

| Entry          | Sequence                      | Expected mass | Observed mass |
|----------------|-------------------------------|---------------|---------------|
| Ligand R\textsubscript{L5} | 3'\text{NH}_2TAUUAUUA-5'    | 2453          | 2453          |
| Ligand R\textsubscript{L6} | 3'-AAAAAUUAU-PO_3^{-2}-5'    | 2582          | Not detected  |
| Template\textsubscript{C}_T3 | 5'-UUUUAAATAUAAUUAU-3'    | 4891          | 4888          |
| Ligation product R\textsubscript{P3} | 3'-AAAAAUUAU(NH)TAUUAUUA-5' | 5018          | 5016          |

Yield (%)  
1 h 4 h 24 h  
19% 49% 61%
**Control experiment:** RDNA chimeric ligands in the absence of any template

\[
5\text{'}-\text{UUUUAAAT}^{\text{NH2}}-3\text{'} + 5\text{'}-\text{PO}_3^{2-}\text{AUAAUAAU}-3\text{'} \xrightarrow{\text{0.2 M EDC, 0.1 M HEPES, pH7.5, 4°C NO TEMPLATE}}
\]

| Entry | Sequence | Expected mass | Observed mass |
|-------|----------|---------------|---------------|
| Ligand C\(_{\text{L3}}\) | 5\text{'}-UUUUAAT^{\text{NH2}} | 2405 | 2405 |
| Ligand C\(_{\text{L4}}\) | 5\text{'}-\text{PO}_3^{2-}\text{AUAAUAAU}-3\text{'} | 2502 | 2502 |
| Ligation product C\(_{\text{P2}}\) | 5\text{'}-UUUUAAT(NH)\text{AUAAUAAU}-3\text{'} | 4890 | Not Observed |

**Supplementary Figure 42.** AEC traces of the control of ligation reaction between heterogeneous RDNA ligands (0.2 mM of each) in absence of any template with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer and ligands. Even after 24 h of reaction no peak corresponding to the ligation product was detected (Top trace). MALDI of the 24 h reaction mixture also confirmed the absence of the ligation product peak (Bottom table). **Gradient:** 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
**Control experiment:** RNA ligands in the absence of any template

\[
5'\text{-UUUAAA} \text{NH}_2\text{-}3' + 5'\text{-PO}_3^2\text{-AUAAAUAAU-}3' \xrightarrow{0.2 \text{ M EDC}, 0.1 \text{ M HEPES, pH 7.5, 4°C}} \text{NO TEMPLATE}
\]

**Supplementary Figure 43.** AEC traces of the control of ligation reaction of homogeneous RNA ligands (0.2 mM of each) in absence of any template with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer and ligands. Even after 24 h of reaction no peak corresponding to the ligation product was detected (Top trace). MALDI of the 24 h reaction mixture also confirmed the absence of the ligation product peak (Bottom table). Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
**Control experiment:** DNA ligands in the absence of template

\[
5'\text{-TTTAAAT}^{\text{NH2}}3' + 5'\text{-PO}_3^{2-}\text{-ATAATA-3'} \xrightarrow{\text{NO TEMPLATE}} 0.2 \text{ M EDC, 0.1 M HEPES, pH 7.5, 4°C}
\]

![Graph showing absorbance over retention time with peaks for Ligands and No Ligation product at 0 h, 1 h, 4 h, and 24 h.]

| Entry          | Sequence                  | Expected mass | Observed mass |
|----------------|----------------------------|---------------|---------------|
| Ligand D_{L1} | 5'\text{-TTTAAAT}^{\text{NH2}} | 2397          | 2396          |
| Ligand D_{L2} | 5'\text{-PO}_3^{2-}\text{-ATAATA-3'} | 2496          | 2495          |
| Ligation product D_{P2} | 5'\text{-TTTAAAT(NH)ATAATAA-3'} | 4876          | Not Observed |

**Supplementary Figure 44.** AEC traces of the control of ligation reaction of homogeneous DNA ligands (0.2 mM of each) in absence of any template with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. Even after 24 h of reaction no peak corresponding to the ligation product was detected (Top trace). MALDI of the 24 h reaction mixture also confirmed the absence of the ligation product peak (Bottom table). Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.
**Control experiment:** RNA ligands in the absence of any template

\[ \text{5'-AUUAUUA}^\text{NH2-3'} + \text{5'-PO}_3^2\text{-AUUUAAAA-3'} \rightarrow \text{NO TEMPLATE} \]

Supplementary Figure 45. AEC traces of the control of ligation reaction of RNA ligands (0.2 mM of each) in absence of any template with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. Even after 24 h of reaction no peak corresponding to the ligation product was detected (Top trace). MALDI of the 24 h reaction mixture also confirmed the absence of the ligation product peak (Bottom table). Gradient: 0 to 40\% Buffer B in 1 min then 40 to 90\% Buffer B in 30 min.
Comparison of rate of ligation of RNA Ligands versus RDNA chimeric ligands versus DNA ligands on a RDNA chimeric template

\[ 5'-UUUAAATT^\text{NH2} \quad \text{PO}_3^2-\text{AUAUUAAU-3'} \]
\[ 3'-\text{AAAUUUA} \quad \text{UAUUUUAA-5'} \]

DNA-RNA chimeric template and RNA ligands

\[ 5'-UUUAAATT^\text{NH2} \quad \text{PO}_3^2-\text{AUAUUAAU-3'} \]
\[ 3'-\text{AAAUUUA} \quad \text{UAUUUUAA-5'} \]

DNA-RNA chimeric template and RNA ligands

\[ 5'-\text{TTTTAAT}^\text{NH2} \quad \text{PO}_3^2-\text{AUAUUAAU-3'} \]
\[ 3'-\text{AAAUUUA} \quad \text{UAUUUUAA-5'} \]

DNA-RNA chimeric template and DNA ligands

Supplementary Figure 46. Comparison of the rate of homogeneous and heterogeneous ligands (0.2 mM each) on RDNA chimeric template (0.1 mM). Rate of ligation with homogeneous ligands (DNA and RNA) are much faster (green and black line) than the corresponding heterogeneous ligands (red line). For conditions of reactions see captions of Figures 36-38. Lines in graph are drawn as guide indicating the trend and are not mathematical curve fitting.
Competitive ligation reaction: RDNA chimeric ligands and RNA ligands on RDNA chimeric template

\[
\begin{align*}
5\text{’}-\text{UUUAAAT}^{\text{NH2}} \text{3’} & \quad 5\text{’}-\text{PO}_3^{2-}\text{-AUAAAUAAU-3’} \\
5\text{’}-\text{UUUAAAT}^{\text{NH2}} \text{3’} & \quad 5\text{’}-\text{PO}_3^{2-}\text{-AUAAAUAAU-3’} \\
\end{align*}
\]

0.2 M EDC, 0.1 M HEPES, pH 7.5, 4°C

Supplementary Figure 47. AEC traces of the ligation reaction of RDNA chimeric ligands and RNA ligands (0.2 mM of each) on RDNA chimeric template (0.1 mM) with 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate the preferential ligation of RNA ligands over chimeric ligands by chimeric RNA template (Top trace). Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min. * indicates the unassigned peaks.
Supplementary Figure 48. MALDI-TOF spectrum of the ligation reaction of RDNA chimeric ligands and RNA ligands (0.2 mM of each) on RDNA chimeric template (0.1 mM) with 0.2M EDC, 0.1M HEPES, pH 7.5 buffer (as shown in supplementary Figure 47) after 24 h of reaction. In MALDI-TOF, no product peak corresponding to heterogeneous ligation product was observed as it was merged with the template peak (confirmed by recording the MADI-TOF of the isolated heterogeneous ligation product peak as indicated in the box).
**Competitive ligation reaction:** RDNA chimeric ligands and DNA ligands on RDNA chimeric template

### Entry

| Ligand D<sub>L1</sub> | 5'-TTTTAAT<sup>NH2</sup>-3' | 2397 | 2397 |
|----------------------|-------------------------------|------|------|
| Ligand D<sub>L2</sub>| 5'-PO<sub>3</sub>²-AUAUAAU-3' | 2496 | 2495 |
| Ligand C<sub>L3</sub>| 5'-UUUUAAT<sup>NH2</sup>-3' | 2405 | 2406 |
| Ligand C<sub>L4</sub>| 5'-PO<sub>3</sub>²-AAAAAU-3' | 2502 | 2503 |
| Template C<sub>T2</sub>| 3'-AAAAUUUUAUUAUUAU-5' | 4893 | 4893 |
| Homogeneous Ligation product D<sub>P1</sub> | 5'-TTTTAAT(NH)ATAATAAT-3' | 4874 | 4876 |
| Yield (%) | 1 h | 4 h | 24 h | 23% 49% 78% |
| Heterogeneous Ligation product C<sub>P2</sub>| 5'-UUUUAAT(NH)AAAAUA-3' | 4890 | Merged with the template peak |
| Yield (%) | 1 h | 4 h | 24 h | 5%< 12% 22% |

**Supplementary Figure 49.** AEC traces of the ligation reaction of RDNA chimeric ligands and DNA ligands on RDNA chimeric template in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer with 0.1 mM template and 0.2 mM of each ligands. The AEC traces indicate the preference of chimeric RDNA template for DNA ligands over chimeric ligands from a mixture of both ligands (Top trace). In MALDI, no product peak corresponding to heterogeneous ligation product was detected as it merged with the template peak (Bottom table) which was confirmed by recording the MALDI of the isolated heterogeneous ligation product peak. **Gradient:** 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.
**Ligation reaction.** short tetrameric RNA ligands on octameric all four nucleobase RDNA chimeric template.

\[
\begin{align*}
5'\text{-GUC}_2^\text{NH2} & \quad \text{PO}_3^{2-}\text{-ACGA-3'} \quad 0.2 \text{ M EDC, 0.1 M HEPES, pH 7.5, 4 }\text{ } ^{\circ}\text{C} \\
3'\text{-CAGA} & \quad \text{TGCU-5'}
\end{align*}
\]

### Table

| Entry          | Sequence                          | Expected mass | Observed mass |
|---------------|-----------------------------------|---------------|---------------|
| Ligand R\text{L7} | 5'-GUC\text{NH2}                | 1199          | 1198          |
| Ligand R\text{L8} | 5'-PO\text{3-ACGA-3'}           | 1326          | 1327          |
| Template C\text{T4} | 3'-CAGATGCU-5'              | 2459          | 2460          |
| Ligation product R\text{P4} | 5'-GTC(\text{NH})ACGA-3' | 2506          | 2508          |
| Yield (%)      | 1 h 3 h 20 h                      | 25% 55% 99%   |

**Supplementary Figure 50.** AEC traces of the ligation reaction of short RNA ligands (0.2 mM of each) on heterogeneous RDNA template (C\text{T4}, 0.1 mM) with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The peak corresponding to the ligation product appeared at higher retention time than the template peak (Top trace). MALDI of the 24 h reaction mixture also confirmed the presence of the ligation product peak (Bottom table). *Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.*
**Ligation reaction.** Short tetrameric chimeric RDNA ligands on octameric all four nucleobase RDNA chimeric template.

![Diagram showing ligation reaction](image)

| Entry          | Sequence                           | Expected mass | Observed mass |
|----------------|------------------------------------|---------------|---------------|
| Ligand C_{L5}  | 5'-G{TCT}^{NH2}                   | 1197          | 1197          |
| Ligand C_{L6}  | 5'-PO_3^{2-}{ACG}A-3'            | 1294          | 1296          |
| Template C_{T4}| 3'-C{AG}AT{G}CU-5'                | 2459          | 2461          |
| Ligation product C_{P3} | 5'-G{TCT}(NH){AC}G_{4}-3' | 2472          | 2474          |

**Supplementary Figure 51:** AEC traces of the ligation reaction of short RDNA ligands (0.2 mM of each) on heterogeneous RDNA template (C_{T4}, 0.1 mM) with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The peak corresponding to the ligation product appeared at higher retention time than the template peak (Top trace). MALDI of the 24 h reaction mixture also confirmed the presence of the ligation product peak (Bottom table). Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.
**Temperature effect and emergence of homogeneous sequence.** Effect of temperature in the emergence of homogeneous Vs chimeric ligation product on chimeric RDNA (A,U/T,G,C) template

\[
\begin{align*}
5'&-\text{GUC}^{\text{NH2}} \hspace{1cm} \text{PO}_3^{2-}\text{-ACGA-3'} \\
3'&-\text{CAGA} \hspace{-0.5cm} \text{TGCU-5'}
\end{align*}
\]

\[
\begin{align*}
5'&-\text{GIC}^{\text{NH2}} \hspace{1cm} \text{PO}_3^{2-}\text{-ACGA-3'} \\
3'&-\text{CAGA} \hspace{-0.5cm} \text{TGCU-5'}
\end{align*}
\]

**Supplementary Figure 52:** Comparison of the rate of ligation between homogeneous RNA (R_{L7}, R_{L8}) and heterogeneous DNA-RNA (C_{L5}, C_{L6}) ligands (0.2 mM of each) on heterogeneous DNA-RNA chimeric template (C_{T4}, 0.1 mM) in 0.2M EDC, 0.1 MHEPES, pH 7.5 at different temperature. The Figure indicates that with increasing temperature, the homogeneous ligation product (R_{P4}) formation wins over the heterogeneous ligation product formation (C_{P3}). Lines in graph are drawn as guide indicating the trend and are not mathematical curve fitting.
**Competitive ligation reaction**: RDNA chimeric ligands, DNA ligands and RNA ligands on RDNA chimeric template

![Diagram of ligation reaction](image)

### Table: Ligation Products

| Entry   | Sequence                                      | Expected mass | Observed mass |
|---------|-----------------------------------------------|---------------|---------------|
| Ligand D1 | 5'-**TTTTAAAT**NH2-3'                         | 2397          | 2398          |
| Ligand D2 | 5'-**PO3^2-ATAATAAT**-3'                     | 2496          | 2497          |
| Ligand C1 | 5'-**UUUUAAT**NH2-3'                         | 2405          | 2406          |
| Ligand C2 | 5'-**PO3^2-AUAAUAUAAU-3'                     | 2502          | 2503          |
| Ligand R1 | 5'-**UUUUAAT**NH2-3'                         | 2453          | 2453          |
| Ligand R2 | 5'-**PO3^2-AUAAUAUAAU-3'                     | 2582          | 2583          |
| Template C2 | 3'-**AAAUUU4UAUU4UU4UU4-5'                  | 4893          | 4893          |
| DNA Ligation product Dp1 | 5'-**TTTTAAAT(NH)ATAATAAT**-3' | 4876          | 4876          |
| Yield (%) | 1 h 4 h 24 h                                 | 10%           | 17%           | 20%           |
| RNA Ligation product Rp1 | 5'-**UUUUAAT(NH)AUAAUAUAAU-3' | 5018          | 5017          |
| Yield (%) | 1 h 4 h 24 h                                 | 20%           | 30%           | 38%           |
| Cross-Ligation product Rdp1 | 5'-**TTTTAAAT(NH)AUAAUAUAAU-3' | 4962          | 4962          |
| Yield (%) | 1 h 4 h 24 h                                 | 27%           | 55%           | 75%           |

**Supplementary Figure 53.** AEC traces of the ligation reaction of RDNA chimeric ligands, DNA ligands and RNA ligands (0.2 mM of each) on RDNA chimeric template (0.1 mM) with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The AEC traces indicated the formation of three main peaks at lower retention time compared to the template peak. The three ligation product peaks were isolated and their MALDI analysis indicate these as DNA ligation product (20%), RDNA cross-ligation product (75%) and RNA ligation product (38%). Screenshot of the isolated MALDI peaks are in the next page. **Gradient**: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.
Supplementary Figure 54. MALDI analysis (screen shots) of the isolated peaks from the reaction of RDNA chimeric ligands, DNA ligands and RNA ligands (0.2 mM of each) on RDNA chimeric template (0.1 mM) in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. (a) DNA ligation product, peak retention time 24.2 min; (b) RDNA cross-ligation product, retention time 25.3 min; (c) RNA ligation product, retention time 26.5 min. Corresponding AEC traces are shown on supporting Figure 53. Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min. Buffer A: 10 mM Na₂HPO₄, pH 11; Buffer B: 10 mM Na₂HPO₄, 1 M NaCl, pH 11.
Competitive ligation reaction: DNA ligands and RNA ligands on RDNA chimeric template

\[
\begin{align*}
5'\text{-}TTTTAAAT^{\text{NH2}-3'} & \quad 5'\text{-}PO_3^2\text{-}ATAATAAT\text{-}3' \quad 0.2 \text{ M EDC, 0.1 M HEPES, pH } 7.5, \text{ 4°C} \\
5'\text{-}UUUUAAAT^{\text{NH2}-3'} & \quad 5'\text{-}PO_3^2\text{-}AUAAAUAAU\text{-}3' & 3'\text{-}AAAAUUUAAAAUAAUAUUA-5'
\end{align*}
\]

Supplementary Figure 55. AEC traces of the ligation reaction of DNA ligands and RNA ligands (0.2 mM of each) on RDNA chimeric template (0.1 mM) with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate the formation of DNA ligation product (14%), RDNA cross-ligation product (72%) and RNA ligation product (31%). Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.
Supplementary Figure 56. AEC traces of the reaction of DNA ligands (D_{L1}, D_{L2}) and RNA ligands (R_{L3}, R_{L4}) (0.2 mM of each) in absence of any template in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. This observation indicates the role of template in cross-ligation product R_{DP} formation. Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.
**Cross-ligation reaction:** RNA\(^{\text{NH2}}\)(R\(_{L3}\)) and PO\(_3\)\(^2-\)-DNA (D\(_{L2}\)) ligands on RDNA chimeric template (C\(_{T2}\))

\[
5'\text{-UUUAAAT}^{\text{NH2}} + \text{PO}_3^{2-}\text{-ATAATAAT-3'}
\]

\[
0.2 \text{ M EDC, 0.1 M HEPES, pH 7.5 , 4°C}
\]

\[
3'\text{-AAAAAUUAAUUAAUAUA-5'}
\]

**Supplementary Figure 57.** AEC traces of the ligation reaction of RNA\(^{\text{NH2}}\) (R\(_{L3}\)) ligands and PO\(_3\)\(^2-\)-DNA (D\(_{L2}\)) ligands (0.2 mM of each) on RDNA chimeric template (0.1 mM) with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer with template and ligands. MALDI of the 24 h reaction mixture confirms the formation of cross-ligation product R\(_{DP2}\) (bottom table). This observation indicates the nature of the cross-ligation product. **Gradient:** 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.

| Entry                     | Sequence                          | Expected mass | Observed mass |
|---------------------------|-----------------------------------|---------------|---------------|
| Ligand R\(_{L3}\)         | 5'-UUUUAAAT\(^{\text{NH2}}\)      | 2453          | 2453          |
| Ligand D\(_{L2}\)         | 5'-PO\(_3\)\(^2-\text{-ATAATAAT-3'}\) | 2496          | 2496          |
| Template C\(_{T2}\)       | 3'-AAAAAUUUAAUUAAUAUA-5'          | 4893          | 4893          |
| Cross-Ligation product R\(_{DP2}\) | 5'-UUUUAAAT(NH)ATATAAT-3'   | 4932          | 4930          |
| Yield (%)                 | 1 h 4 h 24 h                      | 60% 99% 119%  |               |

**Table:**

- **Entry:** Ligand R\(_{L3}\), Ligand D\(_{L2}\), Template C\(_{T2}\), Cross-Ligation product R\(_{DP2}\)
- **Sequence:** 5'-UUUUAAAT\(^{\text{NH2}}\), 5'-PO\(_3\)\(^2-\text{-ATAATAAT-3'}\), 3'-AAAAAUUUAAUUAAUAUA-5', 5'-UUUUAAAT(NH)ATATAAT-3'
- **Expected mass:** 2453, 2496, 4893, 4932
- **Observed mass:** 2453, 2496, 4893, 4930
- **Yield (%):** 60%, 99%, 119%
**Cross-ligation reaction:** DNA\(^{NH2}\) (D\(_{L1}\)) and PO\(_3^{2−}\)–RNA (R\(_{L4}\)) ligands on RDNA chimeric template (C\(_{T2}\))

```
5′-TTTTAAAT\(^{NH2}\) + PO\(_3^{2−}\)-AUAAUAAU-3′  3′-AAAAUUUAUUAUUUAUUA-5′
```

Supplementary Figure 58. AEC traces of the ligation reaction of DNA\(^{NH2}\) (D\(_{L1}\)) ligand and PO\(_3^{2−}\)–RNA (R\(_{L4}\)) ligands on RDNA chimeric template in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer with 0.1 mM template and 0.2 mM of each ligands. MALDI of the 24 h reaction mixture confirms the formation of cross-ligation product R\(_{DP1}\) (bottom table). This observation indicates the nature of the cross-ligation product. Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.
Effect of varying concentrations of DNA and RNA ligands on the product distribution between the RNA ligation products versus the DNA ligation products versus the RNA-DNA cross ligation products.

Supplementary Figure 59. The formation of DNA ligation product (blue), RNA-DNA cross-ligation product (brown) and RNA ligation product (gray) depending on the varied concentration of DNA and RNA ligands on a RDNA chimeric template. Figure A: template and RNA concentrations were fixed (0.1 mM RDNA chimeric template and 0.2 mM RNA ligand) and DNA concentrations were varied (0.04 mM, 0.08 mM and 0.12 mM). With increased concentrations of DNA, more RNA-DNA cross-ligation products were formed. Figure B: template and DNA concentrations were fixed (0.1 mM RDNA chimeric template and 0.2 mM DNA ligand) and RNA concentrations were varied (0.04 mM, 0.08 mM and 0.12 mM). With increased concentrations of RNA, more RNA-DNA cross-ligation products are formed. Experiments were run in triplicate and the error range is less than ± 5%.
**Competitive ligation reaction**: DNA ligands and RNA ligands on RNA template

\[
5'-TTTTAAAT^{NH2-3'} \quad 5'-PO_3^{2-}-ATAATAAT-3' \quad 0.2 \text{ M EDC, } 0.1 \text{ M HEPES, pH7.5 , 4°C}
\]

\[
5'-UUUAAAAT^{NH2-3'} \quad 5'-PO_3^{2-}-AUAAUAU-3' \quad 3'-AAAUUUAUUUAUUUA-5'
\]

![Diagram of ligation products](image)

| Entry          | Sequence                                      | Expected mass | Observed mass |
|----------------|-----------------------------------------------|---------------|---------------|
| DNA Ligation   | 5'-TTTTAAAT(NH)ATAATAAT-3'                    | 4876          | 4876          |
| product Dp1    | 5'-UUUAAAAT(NH)AUAAUAU-3'                    | 5018          | 5016          |
| Yield (%)      | 1 h 4 h 24 h                                 | 22% 51% 65%   |
| RNA Ligation   | 5'-TTTTAAAT(NH)ATAATAAT-3'                    | 4962          | 4961          |
| product Rp2    | 5'-UUUAAAAT(NH)ATAATAAT-3'                    | 4932          | 4930          |
| Yield (%)      | 1 h 4 h 24 h                                 | 21% 45% 62%   |

**Supplementary Figure 60.** AEC traces of the ligation reaction of DNA ligands and RNA ligands on RNA template in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer with 0.1 mM template and 0.2 mM of each ligands. The AEC traces indicate the formation of DNA ligation product (12%), RDNA cross-ligation product (62%) and RNA ligation product (65%) which was confirmed by recording MALDI of the isolated peaks after 24 h of reaction. Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
Characterization of cross-ligation product formation on RDNA and RNA template in the presence of DNA and RNA ligands

**Supplementary Figure 61:** Establishment of the nature of cross-ligation product in presence of both homogeneous DNA and RNA ligands (0.2 mM of each) on respective template (0.1 mM) with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. **Figure a.** Reaction of R₃, R₄, D₉, D₁₀ on C₉ template and MALDI-TOF of the isolated cross-ligation peak indicate it to be R₉P₁ as major. **Figure b.** Reaction of R₃ and D₁₀ on C₉ template and MALDI-TOF of the 24 h reaction mixture indicate it to be R₉P₂. **Figure c.** Reaction of D₉ and R₄ on C₉ template and MALDI-TOF of the 24 h reaction mixture indicates it to be R₉P₁. **Figure d.** Reaction of R₃, R₄, D₉, D₁₀ on R₉ template and MALDI-TOF of the isolated cross-ligation peak indicate it to be the mixture of R₉P₁ and R₉P₂ of close intensity. Both the R₉P₁ and R₉P₂ appear at the same retention time in AEC as observed in the spiking experiment.
Cross-ligation product (RD$_{P1}$) as template in the ligation reaction

\[
5'\text{-TTTTAAAT(NH)\text{-AUAUUAAU-3'}} \quad \text{and} \quad 3'\text{-AAAUAUUA-PO}_3^{2-} + \text{NH}_2\text{TUAUUAU-5'} \quad \text{at 260 nm}
\]

Retention time (min)

| Entry       | Sequence                        | Expected mass | Observed mass |
|-------------|---------------------------------|---------------|---------------|
| Ligand R$_{L6}$ | 5'-PO$_3^{2-}$-AUUUGAA-3'       | 2582          | 2582          |
| Ligand R$_{L5}$ | 5'-AUUUGAAUAT$_{NH2}$-3'       | 2453          | 2453          |
| Template RD$_{P1}$ | 5'-TTTTAAAT(NH)AUUUAAUAA-3'   | 4962          | 4959          |
| Ligation product RD$_{P3}$ | 3'-AAAUAUUAAT(NH)AUUUAAUAA-5' | 5018          | 5015          |
| Yield (%)    | 1 h 4 h 24 h                    | 33% 73% 108%  |

Supplementary Figure 62. AEC traces of the ligation reaction of RNA ligands (0.2 mM each) (R$_{L5}$, R$_{L6}$) on cross-ligation product template RD$_{P1}$ (0.1 mM) in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer with 0.1 mM template and 0.2 mM of each ligands. Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.
**Supplementary Figure 6.3.** The expected DNA-RNA chimeric-system-based replicating cycle. It was anticipated that the addition of ligands (RL5, RL6), complementary to the 1st ligation product (RP2) will release the chimeric template (CT2) due to the preference of homogeneous ligands for homogeneous template and the released chimeric template will continue the replication-by-ligation further.
Supplementary Figure 64. AEC traces of the stepwise replication cycle of RNA ligands on RDNA chimeric template.

(A) The reaction of RL3,RL4 (0.2 mM of each ligands) on DNA-RNA chimeric template CT2 (0.1 mM) produces the 1st ligation product (RP2) (bottom two spectra). Addition of ligands RL5,RL6 (0.42 mM of each in 2 µl new buffer) causes the second ligation reaction to form 2nd ligation product RP3 (spectras 3-6, Top trace). During that reaction the initial ratio of CT2: RL3,RL4 was (1:2) and after RL5,RL6 addition the final ratio of CT2: RL3,RL4, RL5,RL6 became (1:2:2).

(B) The reaction of RL3,RL4 (0.5 mM of each ligands) on DNA-RNA chimeric template CT2 (0.1 mM) produces the 1st ligation product (RP2) (bottom two spectra). Addition of ligands RL5,RL6 (0.42 mM in 2 ul new buffer) causes the second ligation reaction to form 2nd ligation product RP3 (spectras 3'-6', Bottom spectra). During that reaction the initial ratio of CT2: RL3,RL4 was (1:5) and after RL5,RL6 addition the final ratio of CT2: RL3,RL4, RL5,RL6 became (1:5:2).
**Stepwise replication cycle.** % 1\textsuperscript{st} and 2\textsuperscript{nd} ligation product formation in replication cycle of RNA ligands on RDNA chimeric template

| Reaction time (h) | Initial Template (CT2):Ligands (RL3,RL4) (1:2) | Initial Template (CT2):Ligands (RL3,RL4) (1:5) |
|------------------|-----------------------------------------------|-----------------------------------------------|
|                  | % 1\textsuperscript{st} ligation product (RP2) | % 2\textsuperscript{nd} ligation product (RP3) | % 1\textsuperscript{st} ligation product (RP2) | % 2\textsuperscript{nd} ligation product (RP3) |
| 0                | 0                                             | 0                                             | 0                                             | 0                                             |
| 20               | 94                                            | -                                             | 126                                           | -                                             |
| (20+0)           | 97                                            | 0                                             | 126                                           | 0                                             |
| (20+1)           | 98                                            | 21                                            | 130                                           | 43                                            |
| (20+4)           | 97                                            | 37                                            | 154                                           | 80                                            |
| (20+7)           | 98                                            | 48                                            | -                                             | -                                             |
| (20+8)           | -                                             | -                                             | 183                                           | 118                                           |
| (20+24)          | 102                                           | 77                                            | 251                                           | 204                                           |

The reaction was carried out with 0.1 mM template; %ligation product formation was calculated w.r.t. the DNA-RNA chimeric template (CT2) after normalizing with their molar extinction co-efficient.

Supplementary Figure 65. The top table describes the effect of initial DNA-RNA heterogeneous template and RNA ligand concentration in the formation of 1\textsuperscript{st} and second ligation product. Although the RL5,RL6 concentration remain same but the presence of higher concentration of RL3,RL4 (1:5 Vs 1:2) increases the formation of RP3 (204% Vs 77%, bottom entry). The bottom Figure is the graphical presentation of the formation of RP2 and RP3 with different initial concentration of RL3,RL4 (as shown in the table). Line in graphs are drawn as guide to indicate the trend and is not a mathematical curve fitting. Experiments were run in triplicate and the error range is less than ± 5%.
**Control ligation reaction.** Chimeric RDNA (C<sub>T2</sub>) template and its parallel RNA (R<sub>L5</sub>, R<sub>L6</sub>) ligands

![Diagram](image)

| Entry          | Sequence                              | Expected mass | Observed mass |
|----------------|---------------------------------------|---------------|---------------|
| Ligand R<sub>L5</sub> | 5'-PO<sub>3</sub>-AUUUUAAAAA-3'       | 2582          | 2582          |
| Ligand R<sub>L6</sub> | 5'-AUUAUUUA<sub>T</sub>NH2-3'         | 2453          | 2452          |
| Template C<sub>T2</sub> | 3'-AAAAUUUUUAUUUUU-5'                  | 4893          | 4890          |
| Ligation product R<sub>P3</sub> | 5'-AUUUAAAA(NH)AUUUAUUA-3'            | 5018          | Not observed  |

**Supplementary Figure 66.** The AEC traces of the ligation reaction of homogeneous RNA bottom strand ligands R<sub>L5</sub>, R<sub>L6</sub> (0.2 mM each) in presence of DNA-RNA chimeric template C<sub>T2</sub> (0.1 mM) in 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. C<sub>T2</sub> is parallel to R<sub>L5</sub>, R<sub>L6</sub> and the AEC traces indicates that the no R<sub>P3</sub> peak formed (Top). MALDI of the 24 h reaction mixture also confirmed the absence of the ligation product peak (Bottom). Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
Control ligation reaction. The ligation reaction to demonstrate the role of chimeric RDNA template in overcoming the homogeneous-homogeneous template-product inhibition in replication cycle.

\[ [R_{P2}] \quad 5'\text{-UUUUAAAT(NH)---AUAAUAUU-3'} + 5'\text{-UUUUAAATNH2-3'} + 5'\text{-PO}_3^2\text{-AUAAUAU-3'} \]
\[ [R_{P3}] \quad 3'\text{-AAAUUUA---(NH)TAUUAAUA-5'} \]
\[ [R_{L3}] \quad [R_{L4}] \]

No reaction and the T2:T3 ratio remain same even after 24 h of reaction

\[ [R_{P2}] \quad 5'\text{-UUUUAAAT(NH)---AUAAUAUU-3'} + 5'\text{-UUUUAAATNH2-3'} + 5'\text{-PO}_3^2\text{-AUAAUAU-3'} \]
\[ [R_{P3}] \quad 3'\text{-AAAUUUA---(NH)TAUUAAUA-5'} \]
\[ [R_{L3}] \quad [R_{L4}] \]

More \([R_{P2}]\) formed and the \([R_{P2}] : [R_{P3}]\) ratio changes with time

Supplementary Figure 67. The ligation reaction of RNA \((R_{L3}, R_{L4})\) ligands (0.2 mM each) in the presence of two complementary ligation \((R_{P2}, R_{P3})\) products (0.1 mM each) in 0.2 M EDC, 0.1 M HEPES, pH 7.5 buffer. After, 24 h of reaction RDNA chimeric \((C_{T2})\) template was added to the reaction mixture (See caption of Supplementary figure 68).
**Control ligation reaction.** The ligation reaction to demonstrate the role of chimeric RDNA template in overcoming the homogeneous-homogeneous template-product inhibition in replication cycle.

**Supplementary Figure 68.** The experimental demonstration of the role of DNA-RNA chimeric template (C\textsubscript{T2}) in the continuous production of 1\textsuperscript{st} ligation product (R\textsubscript{P2}) in the presence of two complementary homogeneous ligation product R\textsubscript{P2} and R\textsubscript{P3}. The AEC traces indicate the ligation reaction of R\textsubscript{L3}, R\textsubscript{L4} (0.2 mM of each) in presence of R\textsubscript{P2} and R\textsubscript{P3} (0.1 mM of each) in 0.2 M EDC, 0.1 M HEPES, pH 7.5 at 4 °C. The unchanged ratio of R\textsubscript{P2} and R\textsubscript{P3} indicate that no ligation reaction occur even after 24 h of reaction (bottom two spectra). Then the addition of C\textsubscript{T2} changes the R\textsubscript{P2} and R\textsubscript{P3} ratio indicating the ligation reaction. Bottom Figure indicated the change in R\textsubscript{P2} and R\textsubscript{P3} ratio. **Gradient:** 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min. Line in graph are drawn as guide indicating the trend and are not mathematical curve fitting.
One-pot replication cycle. All four RNA ligands on RDNA chimeric template

\[
\begin{align*}
&\text{[R}_{L3}\text{]} \quad 5'\text{-UUUUAAAAT}^\text{NH2-3'} \\
&\text{[R}_{L4}\text{]} \quad 5'\text{-PO}_3^2\text{-AUAAUAU-3'} \\
&\text{[C}_{T2}\text{]} \quad 3'\text{-AAAAUUUUAAUUUUA-5'} \\
&\text{[R}_{L5}\text{]} \quad 5'\text{-AUAAUAUAT}^\text{NH2-3'} \\
&\text{[R}_{L6}\text{]} \quad 5'\text{-PO}_3^2\text{-AUUAAAAA-3'}
\end{align*}
\]

0.2 M EDC, 0.1 M HEPES, pH 7.5, 4°C

Template (C\textsubscript{T2})

1\textsuperscript{st} ligation product (R\textsubscript{P2})

2\textsuperscript{nd} ligation product (R\textsubscript{P3})

Supplementary Figure 69: The AEC traces of the one-pot ligation reaction of R\textsubscript{L3}, R\textsubscript{L4} (0.5 mM of each) R\textsubscript{L5}, R\textsubscript{L6} (0.2 mM of each) on DNA-RNA chimeric template C\textsubscript{T2} (0.1 mM) in 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The AEC traces indicates the appearance of 1\textsuperscript{st} ligation product (R\textsubscript{P2}) in lower retention time than the template and 2\textsuperscript{nd} ligation product (R\textsubscript{P3}) in higher retention time than the template (Top). The second ligation product formation is confirmed by spiking experiment. Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
Control ligation reaction. 0.2 mM all four RNA ligands and no template

**Entry** | **Sequence** |
---|---|
Ligand R<sub>L3</sub> | 5'-UUUAAA<sub>NH2</sub>-3' |
Ligand R<sub>L4</sub> | 5'-PO<sub>3</sub>²-AUAAAUAU-3' |
Ligand R<sub>L5</sub> | 5'-AUUAUUA<sub>NH2</sub>-3' |
Ligand R<sub>L6</sub> | 5'-PO<sub>3</sub>²-AUUUAAAA-3' |
Background reaction product | 24 h |
Yield(%) w.r.t. 0.1 mM external standard dT<sub>24</sub> | Peak-1 Peak-2 Peak-3 |
| 33% 20% 13% |

**Supplementary Figure 70.** The AEC traces of the one-pot reaction containing R<sub>L3</sub>, R<sub>L4</sub>, R<sub>L5</sub>, R<sub>L6</sub> (0.2 mM of each) in absence of any template in 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The AEC traces indicates the appearance of background reaction and the appearance of newer peaks in the ligation product region (Top). Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
Temperature effect in background ligation reaction.

| Entry   | Sequence                                    |
|---------|---------------------------------------------|
| Ligand R_L3 | $5'$-UUUUAAAT^{NH2}-3'$                     |
| Ligand R_L4 | $5'$-PO$_3^2$-AUAAUAU-3'$                  |
| Ligand R_L5 | $5'$-AUUAUAAAT^{NH2}-3'$                  |
| Ligand R_L6 | $5'$-PO$_3^2$-AUUUAAA-3'$                  |

**Supplementary Figure 71.** At different temperature the AEC traces of the 24 h one-pot reaction mixture containing R_L3, R_L4, R_L5, R_L6 (0.2 mM of each) in absence of any template in 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The AEC traces indicates the appearance of newer peaks in the ligation product region (Top). The AEC traces also indicate that with increasing temperature the ligands are decomposed (as indicated by more numbers of peak at the ligands region). Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
Testing EDC effect in background ligation reaction.

Supplementary Figure 72. The AEC traces of the 24 h one-pot reaction mixture containing R₃, R₄, R₅, R₆ (0.2 mM of each) in absence of any template in 0.02M EDC in 0.1M HEPES, 150 mM NaCl pH 7.5 buffer. The AEC traces indicates the appearance of newer peaks in the ligation product region (Top) although lower in intensity compared to 0.2 M EDC, 0.1 M HEPES, pH7.5 buffer. Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
Lowering ligands concentration and the background ligation reaction.

Supplementary Figure 73. The AEC traces of the 24 h one-pot reaction mixture containing RL3, RL4, RL5, RL6 (20 µM of each) in absence of any template in 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The AEC traces indicates that no background reaction by the absence of new peak at the ligation product region. Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
Lowering ligands concentration and the background ligation reaction. 20 µM all four RNA ligands and no template

Supplementary Figure 74. The AEC traces of the 24 h one-pot reaction mixture containing R_{L3}, R_{L4}, R_{L5}, R_{L6} (20 µM of each) in absence of any template in 0.2M EDC in 0.1M HEPES, 150 mM NaCl, pH 7.5 buffer. The AEC traces indicates that no background reaction by the absence of new peaks at the ligation product region. Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
**One-pot replication cycle.** All four RNA ligands (20 µM each) on RDNA chimeric template (10 µM)

\[
\begin{array}{c}
5'\text{-UUUAAAAT}^{\text{NH}_2}3' \\
5'\text{-PO}_3^{2-}\text{-AUAAUUAAU-3'}
\end{array}
\]

\[
\begin{array}{c}
3'\text{-AAAAUUUAUUUAUUU-5'}
\end{array}
\]

\[
\begin{array}{c}
5'\text{-AUUAAUAT}^{\text{NH}_2}3' \\
5'\text{-PO}_3^{2-}\text{-AUUUAAAAA-3'}
\end{array}
\]

**Supplementary Figure 75.** The AEC traces of the one-pot reaction mixture containing R_{L3}, R_{L4}, R_{L5}, R_{L6} (20 µM of each) on DNA-RNA chimeric C_{T2} template (10 µM) in 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The AEC traces indicates both the ligation product although the formation of second ligation product is in minor quantity (Top). Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
**One-pot replication cycle.** All four RNA ligands (20 µM each) on RDNA chimeric template (10 µM)

| Entry | Sequence                                      |
|-------|-----------------------------------------------|
| Ligand R\textsubscript{L3} | 5'-UUUUAAAT\textsuperscript{NH2}-3'         |
| Ligand R\textsubscript{L4} | 5'-PO\textsubscript{3}-AUAAUAAU-3'           |
| Ligand R\textsubscript{L5} | 5'-PO\textsubscript{3}-AUUUUAAA-3'           |
| Ligand R\textsubscript{L6} | 5'-AUUAUUAA\textsuperscript{NH2}-3'          |
| Template C\textsubscript{T2} | 3'-AAAUUUAUUUAAUUA-5'                      |

**Supplementary Figure 76.** The AEC traces of the one-pot reaction mixture containing R\textsubscript{L3}, R\textsubscript{L4}, R\textsubscript{L5}, R\textsubscript{L6} (20 µM of each) on DNA-RNA chimeric C\textsubscript{T2} template (10 µM) in 0.2M EDC in 0.1M HEPES, 150 mM NaCl, pH 7.5 buffer. The AEC traces indicates both the 1\textsuperscript{st} ligation product and 2\textsuperscript{nd} ligation product after 24 h of reaction (Top trace). *Gradient:* 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
**One-pot replication cycle.** All RNA (R<sub>L3</sub>, R<sub>L4</sub>, R<sub>L5</sub>, R<sub>L6</sub>) ligands and RDNA chimeric (C<sub>L3</sub>,C<sub>L4</sub>) ligands on RDNA chimeric template.

\[
\begin{align*}
5'-\text{UUUUAAAT}^{\text{NH2}-3'}_{[C_{L3}]} & \quad + \quad 5'-\text{PO}_3^2-\text{AUAAUUAAU-3'}_{[C_{L4}]} & \text{0.2 M EDC,} \\
5'-\text{UUUUAAAT}^{\text{NH2}-3'}_{[R_{L3}]} & \quad + \quad 5'-\text{PO}_3^2-\text{AUAAUUAAU-3'}_{[R_{L4}]} & \text{0.1 M HEPES,} \\
3'-\text{AAAAUUUAUUUAUUAA-5'}_{[C_{T2}]} & & \text{150 mM NaCl,} \\
5'-\text{AUUAUUAAAT}^{\text{NH2}-3'}_{[R_{L5}]} & \quad + \quad 5'-\text{PO}_3^2-\text{UUUAAA-3'}_{[R_{L6}]}
\end{align*}
\]

**Supplementary Figure 77.** The AEC traces of the one-pot reaction mixture containing R<sub>L3</sub>,R<sub>L4</sub>, C<sub>L3</sub>,C<sub>L4</sub>,R<sub>L5</sub>,R<sub>L6</sub> (20 µM of each) on DNA-RNA chimeric C<sub>T2</sub> template (10 µM) in 0.2M EDC in 0.1M HEPES, 150 mM NaCl, pH 7.5 buffer. The AEC traces indicates both the 1<sup>st</sup> ligation product and 2<sup>nd</sup> ligation product after 24 h of reaction (Top trace). It also indicates no sign of inhibition in 2<sup>nd</sup> ligation product formation. **Gradient:** 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.

| Entry | Sequence | Yield (%) |
|-------|----------|-----------|
| Ligand R<sub>L3</sub> | 5'-UUUUAAAT<sup>NH2</sup>-3' | 83% (24 h), 103% (48 h) |
| Ligand R<sub>L4</sub> | 5'-PO<sub>3</sub><sup>2-</sup>-AUAAUUAAU-3' | |
| Ligand C<sub>L3</sub> | 5'-UUUUAAAT<sup>NH2</sup>-3' | |
| Ligand C<sub>L4</sub> | 5'-PO<sub>3</sub><sup>2-</sup>-AUAAUUAAU-3' | |
| Ligand R<sub>L5</sub> | 5'-PO<sub>3</sub><sup>2-</sup>-UUUAAA-3' | |
| Ligand R<sub>L6</sub> | 5'-AUUAUUAAAT<sup>NH2</sup>-3' | |
| Template C<sub>T2</sub> | 3'-AAAAUUUAUUUAUUAAUA-5' | |
| 1<sup>st</sup> Ligation product R<sub>P2</sub> | 5'-UUUUAAAT(NH)AUAAUUAAU-3' | |
| Yield (%) | 83% | 103% |
| 2<sup>nd</sup> Ligation product R<sub>P3</sub> | 3'-AAAAUUUA(UH)AUAAUUUA-3' | |
| Yield (%) | 16% | 31% |
Variable ligands effect. Comparison of the formation of $R_{P2}$ and $R_{P3}$ on RNA $R_{T2}$ and chimeric RDNA $C_{T2}$ template in 48 h of one-pot self-replication cycle with 10 µM template and varied ligands concentrations.

| Ligation product | Template: $R_{L3}, R_{L4}: R_{L5}, R_{L6}$ | $[R_{T2}]$ RNA Template | $[C_{T2}]$ DNA-RNA Chimeric Template |
|------------------|---------------------------------|------------------|---------------------------------|
| 1st Ligation product $R_{P2}$ | 1:2:2 | 99% | 109% |
| % Yield | 1:5:2 | 119% | 178% |
| | 1:10:2 | 160% | 210% |
| | 1:2:5 | 112% | 94% |
| 2nd Ligation product $R_{P3}$ | 1:2:2 | 18% | 30% |
| % Yield | 1:5:2 | 43% | 77% |
| | 1:10:2 | 55% | 92% |
| | 1:2:5 | 27% | 50% |

Supplementary Figure 78. Graphical presentation of the 1st ($R_{P2}$, Top) and 2nd ($R_{P3}$, second from top) ligation product formation on DNA-RNA chimeric ($C_{T2}$) and RNA template ($R_{P2}$) with different template and ligands ratio. The reaction was carried out with in one-pot with 10 µM template concentration in 0.2 M EDC, 0.1 M HEPES, 150 mM NaCl, pH 7.5 at 4 °C. Experiments were run in triplicate and the error range is less than ± 5%.
Design of short (A,U/T,G,C) RDNA chimeric sequence-based replicating system:

**Supplementary Figure 79.** All four-nucleobase containing octameric DNA-RNA chimeric system based replicating cycle. It is envisaged that the addition of ligands (R₁⁷, R₁₈), complementary to the 1ˢᵗ ligation product (R₄) will release the chimeric template (C₄) due to the preference of homogeneous ligands for homogeneous template and the released chimeric template will continue the cycle further.
**Supplementary Figure 80.** Chimeric RDNA octamer template containing all four nucleobases also shows ability to overcome template-product inhibition. (a) Octameric (AU/TG)-RDNA template CT4 with RNA ligands RL7, RL8 produces RP4, which in the presence of RL9, RL10 forms RP5 and releases the CT4 for another round of ligation reaction. (b) Time course of the EDC-mediated-ligation experiments documenting the dynamic nature of the system. See supplementary figs. 82-84 for EDC-ligation conditions. A, U = RNA; A, T = DNA. Lines in graph (80b) are drawn as guide indicating the trend and are not mathematical curve fittings. Experiments were run in triplicate and the error range is less than ±5%. The stepwise addition of ligands (RL7+RL8, followed by RL9+RL10) to the template (CT4) at 4°C led to the formation of the first ligation product (RP4) followed by the second ligation (RP5) product. Moreover, the formation and presence of the second ligation product (RP5) in the above mixture resulted in an increase in the amount of the first ligation product (RP4) suggesting a positive feedback for each other’s production, again affirming the ability of the chimeric-template CT4 to overcome the product-inhibition problem (Fig. 80b). With longer reaction times (48 h) another peak was observed in AEC traces (Supplementary Figs. 81,85-86) which was determined to be cross ligation products between RL8+RL9 and RL7+RL10. Appropriate control experiments showed the necessity of all components to be present for the reaction to proceed in robust manner (Supplementary Figs. 89-90). As with the previous hexadecameric-system, when all four ligands are present (0.2 mM each) in the absence of template CT4, the background reaction was very slow and predominantly producing the cross-ligation products (Supplementary Figs. 87-88) and could be eliminated by dialing down the concentration of ligands to 20 µM each. To probe the dynamic behavior of this system, we prepared a fresh reaction mixture and at 72 h divided it into two parts. To part 1, we added more RL7+RL8 and after 48 h followed by RL9+RL10, whereas to part 2 we reversed the sequence of addition (RL9+RL10 followed, at 48 h, by RL7+RL8) and monitored the progress of the ligation reactions (Supplementary Figs. 83-84). In both cases, the continuous and sequential increase of respective products RP4 and RP5 was observed (Fig. 80b) and was accompanied by formation of cross-ligation products (RL7+RL10 and RL9+RL8, Supplementary Figs. 85-86).
Short sequence replication cycle. Development of short (A,U/T,G,C) RDNA chimeric template-based replication cycle.

Supplementary Figure 81: The AEC traces of the heterogeneous DNA-RNA template mediated emergence of homogeneous sequences. Similar to the hexadecameric A,U template (C\textsubscript{T4}), the template as short as octameric (C\textsubscript{T4}), also able to complete the replicating cycle. Bottom two traces (0 h and 20 h) indicates the emergence of 1\textsuperscript{st} ligation product (R\textsubscript{P4}) in presence of homogeneous ligands (R\textsubscript{L7},R\textsubscript{L8}) on heterogeneous template (C\textsubscript{T4}, 0.1 mM). After 20 h of reaction two homogeneous ligands (R\textsubscript{L9},R\textsubscript{L10}) complementary to R\textsubscript{P4} was added and the reaction was monitored up to 48 h and the AEC traces indicates the appearance of 2\textsuperscript{nd} ligation product (R\textsubscript{P5}) beside initial template (C\textsubscript{T4}) and 1\textsuperscript{st} ligation product (R\textsubscript{P4}) (top five spectra). During that reaction the final ratio of C\textsubscript{T4}: R\textsubscript{L7},R\textsubscript{L8}: R\textsubscript{L9},R\textsubscript{L10} was (1:2:2). \textit{Gradient: 0 to 20\% Buffer B in 1 min then 20 to 90\% Buffer B in 30 min.}

| Entry   | Sequence present | % R\textsubscript{P4} formed | % R\textsubscript{P5} formed | % cross-ligation product |
|---------|------------------|-----------------------------|-----------------------------|--------------------------|
| 0 h     | C\textsubscript{T4},R\textsubscript{L7},R\textsubscript{L8} | 0                           | -                           | -                        |
| 20 h    | C\textsubscript{T4},R\textsubscript{P4},R\textsubscript{L7},R\textsubscript{L8} | 99                          | -                           | -                        |
| (20+0) h| C\textsubscript{T4},R\textsubscript{P4},R\textsubscript{L7},R\textsubscript{L8}, R\textsubscript{L9},R\textsubscript{L10} | 99                          | 0                           | 0                        |
| (20+1) h| C\textsubscript{T4},R\textsubscript{P4}, R\textsubscript{P5},R\textsubscript{L7},R\textsubscript{L8}, R\textsubscript{L9},R\textsubscript{L10} | 99                          | 16                          | 0                        |
| (20+2) h| C\textsubscript{T4},R\textsubscript{P4}, R\textsubscript{P5},R\textsubscript{L7},R\textsubscript{L8}, R\textsubscript{L9},R\textsubscript{L10} | 110                         | 25                          | 0                        |
| (20+3) h| C\textsubscript{T4},R\textsubscript{P4}, R\textsubscript{P5},R\textsubscript{L7},R\textsubscript{L8}, R\textsubscript{L9},R\textsubscript{L10} | 110                         | 27                          | 0                        |
| (20+20) h| C\textsubscript{T4},R\textsubscript{P4}, R\textsubscript{P5},R\textsubscript{L7},R\textsubscript{L8}, R\textsubscript{L9},R\textsubscript{L10} | 141                         | 64                          | 0                        |
| (20+48) h| C\textsubscript{T4},R\textsubscript{P4}, R\textsubscript{P5},R\textsubscript{L7},R\textsubscript{L8}, R\textsubscript{L9},R\textsubscript{L10} | 172                         | 86                          | 16                       |
**Supplementary Figure 82**: Schematic representation of the dynamic nature of the DNA-RNA octameric heterogeneous template based replicating cycle where the ligands R₇, R₈ were continuously feeding to check the dynamic nature of the cycle. The reaction was started with R₇, R₈ (0.2 mM each) and template (C₄, 0.1 mM). After 20 h, it shows the formation of 1st ligation product (R₄) and to that 20 h old reaction mixture ligands R₉, R₁₀ (complementary to 1st ligation product) were added and the reaction was allowed for another 72 h and then divided into two parts— in one part R₇, R₈ and in another part R₉, R₁₀ were added. The progress of the reaction was monitored by AEC and is presented by supplementary figs. 83 & 84). During that reaction the formation of cross-ligation product between R₇, R₁₀ and R₈, R₉ were also observed. **Continuity of replication cycle.** Testing the dynamic nature of the short sequence-based replication cycle.
Continuity of part-1 of the replication cycle on RDNA template

| Step               | Ligation product | Time  | %Yield |
|--------------------|------------------|-------|--------|
| Step I             |                  |       |        |
| Time               |                  | 20 h  |        |
| %Yield             |                  | 101%  |        |
| Step II            | R_p4             |       |        |
| Time               | R_p5             | 72 h  |        |
| %Yield             | Cross            | 167%  | 112%   |
| Step III, Part-1   | R_p4             |       |        |
| Time               | R_p5             | 48 h  |        |
| %Yield             | Cross            | 272%  | 151%   |
| Step IV, Part-1    | R_p4             |       |        |
| Time               | R_p5             | 20 h  |        |
| %Yield             | Cross            | 292%  | 229%   |

Supplementary Figure 83. The AEC profile of the part-1 of the diagram as indicated in supplementary Figure 82. This peak was characterized as the cross-ligation product peak between R_L7,R_L9 and R_8,R_L10 (as confirmed by supplementary figs. 85 and 86. Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.
Continuity of part-2 of the replication cycle on RDNA template

| Step | Ligation product | Time | %Yield |
|------|------------------|------|--------|
| Step I | R_P4 | 20 h  | 101%  |
| Step II | R_P4, R_P5, Cross | 72 h  | 167%, 112%, 41% |
| Step III, Part-2 | R_P4, R_P5, Cross | 48 h  | 201%, 182%, 72% |
| Step IV, Part-2 | R_P4, R_P5, Cross | 20 h  | 306%, 197%, 106% |

**Supplementary Figure 84.** The AEC profile of the part-2 of the diagram as indicated in supplementary Figure 82. The Figure indicates that after 72 h, along with the R_P4 and R_P5, a new peak appears. This peak was characterized as the cross-ligation product peak between R_{L7, R_{L9}} and R_{L8, R_{L10}} (as confirmed by supplementary figs. 85 and 86). Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.
**Cross Ligation reaction:** RNA (R_{L9}, R_{L8}) ligands on chimeric RDNA C\textsubscript{T4} template

\[
5'-\text{UCG}_2^N\text{H}_2 + \text{PO}_3^{2-}\text{-ACGA}-3' \quad 0.2 \text{ M EDC, } 0.1 \text{ M HEPES, pH 7.5, 4 °C}
\]

\[
3'\text{-CAGATGCU-5'} \quad \text{C}\text{T4}
\]

**Supplementary Figure 85:** The AEC traces of the cross-ligation reaction between homogeneous RNA (R_{L8}, R_{L9}) ligands (0.2 mM each) in presence of DNA-RNA heterogeneous (C\textsubscript{T4}) template (0.1 mM) with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The peak corresponding to the cross-ligation product appeared at higher retention time than the template peak (Top trace). MALDI of the 24 h reaction mixture also confirmed the presence of the ligation product peak (Bottom table).

**Gradient:** 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.

| Entry | Sequence | Expected mass | Observed mass |
|-------|----------|---------------|---------------|
| Ligand R_{L9} | 5'-UCG\textsubscript{2}^N\text{H}_2 | 1199 | 1199 |
| Ligand R_{L8} | 5'-\text{PO}_3^{2-}\text{-ACGA}-3' | 1326 | 1328 |
| Template C\textsubscript{T4} | 3'\text{-CAGATGCU-5'} | 2459 | 2461 |
| Cross-Ligation product | 5'-\text{UCG}_2(NH)\text{ACGA}-3' | 2506 | 2508 |
| Yield (%) | 24 h | 134% |
Cross Ligation reaction: RNA (RL7,RL10) ligands on chimeric RDNA C_{T4} template

\[
\begin{align*}
5'{-}\text{GUC}^\text{NH2} + \text{PO}_3^{2-} -\text{AGAC} - 3' \\
[\text{RL7}] & \rightarrow 0.2 \text{ M EDC, 0.1 M HEPES, pH 7.5, 4 °C} \\
3'{-}\text{CAGATGCU-5'} \\
[\text{C}_{T4}] &
\end{align*}
\]

### Absorbance (mAU) at 260 nm vs. Retention time (min)

- **0 h**: Initial conditions
- **1 h**: One hour after initiation
- **3 h**: Three hours after initiation
- **24 h**: Twenty-four hours after initiation

### Table

| Entry        | Sequence                  | Expected mass | Observed mass |
|--------------|---------------------------|---------------|---------------|
| Ligand RL7   | 5'-GUC{\text{NH2}}       | 1199          | 1198          |
| Ligand RL10  | 5'-PO_3^{2-} -AGAC-3'     | 1326          | 1327          |
| Template C_{T4} | 3'-CAGATGCU-5'  | 2459          | 2460          |
| Ligation product | 5'-GUC{(NH)}AGAC-3' | 2506          | 2506          |
| Yield (%)    |                           | 24 h          | 64%           |

**Supplementary Figure 86**: The AEC traces of the cross-ligation reaction between homogeneous RNA (RL7,RL10) ligands (0.2 mM each) in presence of DNA-RNA heterogeneous (C_{T4}) template (0.1 mM) with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The peak corresponding to the cross-ligation product appeared at higher retention time than the template peak (Top trace). MALDI of the 24 h reaction mixture also confirmed the presence of the ligation product peak (Bottom table). *Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.*
Background ligation reaction in short sequence.

\[
\begin{align*}
5'&\text{-GUC}_2\text{NH}_2\text{-3'} & 5'&\text{-PO}_3\text{2-ACGA-3'} & 0.2\text{M EDC, 0.1M HEPES, pH 7.5, 4 }^\circ\text{C} \\
3'&\text{-CAGA-PO}_3\text{2-3'} & 3'&\text{NH}_2\text{IGCU-5'}
\end{align*}
\]

![AEC traces of the one-pot reaction containing R\textsubscript{L7}, R\textsubscript{L8}, R\textsubscript{L9}, R\textsubscript{L10} (0.2 mM of each) in absence of any template in 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The AEC traces indicates the occurrence of background reaction and the appearance of newer peaks in the ligation product region (Top trace). Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.](image)

| Entry        | Sequence          | Expected mass | Observed mass |
|--------------|-------------------|---------------|---------------|
| Ligand R\textsubscript{L7} | 5'\text{-GUC}\text{2}\text{NH}_2\text{-3'} | 1199          | 1198          |
| Ligand R\textsubscript{L8} | 5'\text{-PO}_3\text{2-ACGA-3'} | 1326          | 1327          |
| Ligand R\textsubscript{L10} | 3'\text{-CAGA-PO}_3\text{2-5'} | 1326          | 1327          |
| Ligand R\textsubscript{L9} | 3'\text{NH}_2\text{IGCU-5'} | 1199          | 1199          |
| Template     | No Template       | -             | -             |
| Background   | Ligation product  | -             | Not measured as the combinations have same mass |

Supplementary Figure 87. The AEC traces of the one-pot reaction containing R\textsubscript{L7}, R\textsubscript{L8}, R\textsubscript{L9}, R\textsubscript{L10} (0.2 mM of each) in absence of any template in 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The AEC traces indicates the occurrence of background reaction and the appearance of newer peaks in the ligation product region (Top trace). Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.
Background ligation reaction in short sequence.

![AEC traces of the one-pot reaction mixture containing R_{L3}, R_{L4}, R_{L5}, R_{L6} (20 µM of each) in absence of any template in 0.2M EDC in 0.1M HEPES, 150 mM NaCl, pH 7.5 buffer. The AEC traces indicates that no background reaction (Top). Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.](image)

| Entry | Sequence | Expected mass | Observed mass |
|-------|----------|---------------|---------------|
| Ligand R_{L7} | 5'-GUC{NH}_2^{3'} | 1199 | 1198 |
| Ligand R_{L8} | 5'-PO_3^{2-}-ACGA-3' | 1326 | 1327 |
| Ligand R_{L10} | 3'-CAGA-PO_3^{2-}-5' | 1326 | 1327 |
| Ligand R_{L9} | 3'-NH_2TGCU-5' | 1199 | 1199 |
| Template | No Template | - | - |
| Background Ligation product | - | - | Not observed |

**Supplementary Figure 88.** The AEC traces of the one-pot reaction mixture containing R_{L3}, R_{L4}, R_{L5}, R_{L6} (20 µM of each) in absence of any template in 0.2M EDC in 0.1M HEPES, 150 mM NaCl, pH 7.5 buffer. The AEC traces indicates that no background reaction (Top). Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.
Control reaction. Homogeneous RNA ligands (R_{L7}, R_{L8}) in absence of any template

\[ 5'\text{-GUC}^{\text{NH2}} + \text{PO}_3^{2-}\text{-ACGA-3'} \quad 0.2 \text{ M EDC, 0.1 M HEPES, pH 7.5, 4 °C} \]

| Entry | Sequence | Expected mass | Observed mass |
|-------|----------|---------------|---------------|
| Ligand R_{L7} | 5'\text{-GUC}^{\text{NH2}} | 1199 | 1199 |
| Ligand R_{L8} | 5'\text{-PO}_3^{2-}\text{-ACGA-3'} | 1326 | 1328 |
| Ligation product R_{P4} | 5'\text{-GUC}^{\text{(NH)}}\text{ACGA-3'} | 2506 | 2507 (in AEC not observed) |

**Supplementary Figure 89:** AEC traces of the control of ligation reaction of homogeneous RNA ligands (0.2 mM of each) in absence of any template with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer and ligands. Even after 24 h of reaction no peak corresponding to the ligation product was detected (Top). Although in the MALDI of the 24 h reaction mixture a small peak corresponds to the ligation product was observed. Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.
Control reaction. Heterogeneous RDNA ($C_{L5},C_{L6}$) ligands in absence of any template

\[ 5'\text{-}G\text{IC}^{\text{NH2}} + \text{PO}_{3}^{2-}\text{-}A\text{CGA} \text{-}3' \rightarrow 0.2 \text{ M EDC, 0.1 M HEPES, pH 7.5, 4 \text{oC}} \]

![Diagram of ligands and absorbance](image)

**Table:**

| Entry          | Sequence                  | Expected mass | Observed mass |
|----------------|---------------------------|---------------|---------------|
| Ligand $C_{L5}$ | $5'\text{-}G\text{ICT}^{\text{NH2}}$ | 1197          | 1195          |
| Ligand $C_{L6}$ | $5'\text{-}\text{PO}_{3}^{2-}\text{-}A\text{CGA} \text{-}3'$ | 1294          | 1294          |
| Ligation product $C_{P3}$ | $5'\text{-}G\text{ICT(NH)ACGA} \text{-}3'$ | 2472          | 2471 (In AEC not observed) |

**Supplementary Figure 90:** AEC traces of the control of ligation reaction of chimeric DNA-RNA ligands (0.2 mM of each) in absence of any template with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer and ligands. Even after 24 h of reaction no peak corresponding to the ligation product was detected (Top). Although in the MALDI of the 24 h reaction mixture a small peak corresponds to the ligation product was observed. *Gradient: 0 to 20\% Buffer B in 1 min then 20 to 90\% Buffer B in 30 min.*
Supplementary Figure 91. The effect of stepwise dilution on RDNA (C_T2) template in the presence of R_L3, R_L4, R_L5, R_L6 ligands.

Stepwise dilution reaction. The effect of stepwise dilution on RDNA (C_T2) template in the presence of R_L3, R_L4, R_L5, R_L6 ligands:

\[
\begin{align*}
5'\text{-UUUUAAAT}^{\text{NH}_2} & \quad \text{PO}_3^{2-}\text{-AUAAUAAU-3'} \\
\text{R}_L3 & \quad \text{R}_L4
\end{align*}
\]

\[
3'\text{-AAAAUUUAUUAAUUAU-5'}
\]

\[
3'\text{-AAAAUUUAUPO}_3^{2-}\text{-NH}_2\text{TAUUAUUA-5'}
\]

0.2 M EDC, 0.1 M HEPES, 150 mM NaCl, pH 7.5, 4°C

Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.

Lines in graph are drawn as guide indicating the trend and are not mathematical curve fitting. Experiment was running triplicate and the error is less than ±5%. 

The reaction was started with 10 µM template and at 24 h interval 1 µL was taken out and the reaction mixture was diluted with 1 µL of reaction buffer containing R_L3, R_L4, R_L5, R_L6 (20 µM of each), so that the ligands concentration in the reaction mixture remain fixed but the template concentration was decreased. All the experiments were at least triplicated, and the yields were calculated w.r.t. the externally added 10 µM dT_{24}. Yield drop is indicative of dilution step.
**Stepwise dilution reaction.** The effect of stepwise dilution on RNA (R_{T2}) template in the presence of R_{L3}, R_{L4}, R_{L5}, R_{L6} ligands.

![Diagram](image)

**Supplementary Figure 9.** The effect of stepwise dilution on RNA R_{T2} template. The reaction was started with 10 µM template and at 24 h interval 1 µL was taken out and the reaction mixture was diluted with 1 µL of reaction buffer containing R_{L3}, R_{L4}, R_{L5}, R_{L6} (20 µM of each), so that the ligands concentration in the reaction mixture remain fixed but the template concentration was decreased. All the experiments were at least triplicated, and the yields were calculated w.r.t. the externally added 10 µM dT_{24}. Yield drop is indicative of dilution step. Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min. Lines in graph are drawn as guide indicating the trend and are not mathematical curve fitting. Experiment was running triplicate and the error is less than ±5%.
**Control reaction for stepwise dilution reaction.** In the presence of $R_{L3}, R_{L4}, R_{L5}, R_{L6}$ ligands only i.e. in the absence of any template under otherwise identical condition.

![Supplementary Figure 93](image)

**Supplementary Figure 93.** Control reaction of the stepwise dilution reaction. The reaction was performed in the presence of $R_{L3}, R_{L4}, R_{L5}, R_{L6}$ (20 µM of each) only (in absence of any template) by keeping all other conditions same. At 24 h interval 1 µL was taken out and the reaction mixture was diluted with 1 µL of reaction buffer containing $R_{L3}, R_{L4}, R_{L5}, R_{L6}$ (20 µM of each), so that the ligands concentration in the reaction mixture remain same. **Gradient:** 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min. Note: the trace at 96 h got truncated at 30 min and, therefore, does not show the dT$_{24}$ peak.
Supplementary Figure 94: The HPLC traces of the one-pot ligation reaction of R₃₂₃·R₃₄·C₅₅·C₈₅·C₉₅·C₁₀₅ with R₃₂₃·R₃₄·C₅₅·C₈₅ and C₇₈·C₆₁ ligands showed the increasing formation of R₃₂₃·R₃₄·C₅₅·C₈₅·C₉₅·C₁₀₅-Regeneration of template with phosphoramidate linkage (C₉₅·C₁₀₅) (Top). The control experiment in the absence of C₇₈·C₆₁ ligands showed the leveling of the formation after 24 h (Bottom table) while the presence of C₇₈·C₆₁ ligands showed the increasing formation of R₃₂₃·R₃₄·C₅₅·C₈₅·C₉₅·C₁₀₅. Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.

| Entry | Ligation product R₂₄ | Regeneration of template with phosphoramidate C₉₅·C₁₀₅ | Yield (%) |
|-------|---------------------|-----------------------------------------------------|-----------|
| 5'-AUUUAA(NH)₃AUUUAA-₃' | 24 h 48 h 72 h | 24 h 48 h 72 h | 108% 125% 148% |
| 3'-AAAAUUUU-PO₃⁻ | 24 h 48 h 72 h | 24 h 48 h 72 h | 108% 125% 148% |
| C₅₅·C₈₅ | 24 h 48 h 72 h | 24 h 48 h 72 h | 108% 125% 148% |
| C₅₅·C₈₅ | 24 h 48 h 72 h | 24 h 48 h 72 h | 108% 125% 148% |
| C₇₈·C₆₁ | 24 h 48 h 72 h | 24 h 48 h 72 h | 108% 125% 148% |

Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
**Enzymatic Ligation reaction.** T4 DNA Ligase mediated ligation reaction of DNA ligands (D_{L1}, D_{L2}) on RDNA chimeric template (C_{T2})

\[
\begin{align*}
5'\text{-TTTTAAAT}^\text{OH-3'} & \quad 5'\text{-PO}_3^2\text{-ATAATAAT-3'} \\
3'\text{-AAAAUUUA} & \quad \text{UAAUUUAA-5'}
\end{align*}
\]

| Entry | Sequence | Expected mass | Observed mass |
|-------|----------|---------------|---------------|
| Ligand D_{L3} | 5'-TTTTAAAT^OH-3' | 2398 | 2401 |
| Ligand D_{L2} | 5'-PO_3^2-ATAATAAT-3' | 2496 | 2498 |
| Template C_{T2} | 3'-AAAAUUUA_UAAUUUAA-5' | 4893 | 4894 |
| Ligation product D_{P2} | 5'-TTTTAAATATAATAAT-3' | 4877 | 4878 |

Yield (%)

| Time | Yield (%) |
|------|-----------|
| 1 h  | <5%       |
| 16 h | 24%       |

**Supplementary Figure 95:** AEC traces of the ligation reaction of DNA ligands (20 µM of each) on DNA-RNA chimeric C_{T2} template (10 µM) in presence of T4 DNA ligase at 4 °C. After 24 h of reaction peak corresponding to the ligation product was detected (Top) which was further confirmed by the MALDI-TOF of the 24 h reaction mixture. *Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.*
**Enzymatic Ligation reaction.** T4 DNA Ligase mediated ligation reaction of DNA ligands (D<sub>L1</sub>, D<sub>L2</sub>) on DNA template (D<sub>T1</sub>)

![Diagram of ligation reaction](image)

| Entry       | Sequence                                | Expected mass | Observed mass |
|-------------|-----------------------------------------|---------------|---------------|
| Ligand D<sub>L3</sub> | 5′-*TTTTAAAT*<sub>OH</sub>-3′            | 2398          | 2401          |
| Ligand D<sub>L2</sub> | 5′-*PO<sub>3</sub>²-*ATAATAAT*-3′       | 2496          | 2499          |
| Template D<sub>T1</sub> | 3′-*AAAAATTTATATTATTA*-5′               | 4877          | 4881          |
| Ligation product D<sub>P2</sub> | 5′-*TTTTAAATATAATAAT*-3′               | 4877          | 4881          |
| Yield (%)   |                                         |               | (both template and ligation product have same mass) |
| 0 min       |                                         | 50%           | 72%           |
| 30 min      |                                         | 72%           | 74%           |
| 60 min      |                                         | 74%           |               |

**Supplementary Figure 96:** AEC traces of the ligation reaction of DNA ligands (10 µM of each) on DNA D<sub>T1</sub> template (10 µM) in presence of T4 DNA ligase at 4 °C. After 24 h of reaction peak corresponding to the ligation product was detected (Top) which was further confirmed by the MALDI-TOF of the 24 h reaction mixture. *Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.*
Enzymatic Ligation reaction. T4 DNA Ligase mediated ligation reaction of RNA ligands (RL11, RL4) on DNA template (DT1)

Supplementary Figure 97: AEC traces of the ligation reaction of RNA ligands (10 µM of each) on DNA (DT1) template (10 µM) in presence of T4 DNA ligase at 4 °C. After 24 h of reaction no peak corresponding to the ligation product was detected (Top) which was further confirmed by the MALDI-TOF of the 24 h reaction mixture. Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
**Enzymatic Ligation reaction.** T4 RNA Ligase2 mediated ligation reaction of RNA ligands ($R_{L11}, R_{L4}$) on RNA template ($R_{T2}$)

![Diagram of ligation reaction](image)

| Entry          | Sequence                        | Expected mass | Observed mass |
|----------------|---------------------------------|---------------|---------------|
| Ligand $R_{L11}$ | 5'-UUUUAAAU$^{OH-3'}$           | 2456          | 2458          |
| Ligand $R_{L4}$  | 5'-PO$_3^{2-}$-AUAAUAAU-3'      | 2582          | 2584          |
| Template $R_{T2}$ | 3'-AAAAAUUAUAUAUAUUA-5'         | 5021          | 5023          |
| Ligation product $R_{P6}$ | 5'-UUUUAAAUAUAUAUAUA-3'      | 5021          | 5023          |
| Yield (%)       | 0 min   30 min   60 min            | 28%           | 84%           |

**Supplementary Figure 98:** AEC traces of the ligation reaction of RNA ligands (10 µM of each) on RNA ($R_{T2}$) template (10 µM) in presence of T4 RNA ligase 2 at 4 °C. The peak corresponding to the ligation product was observed (Top) which was further confirmed by the MALDI-TOF of the 1 h reaction mixture. *Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.*
**Enzymatic Ligation reaction.** T4 RNA Ligase2 mediated ligation reaction of RNA ligands (R_{L1}, R_{L4}) on chimeric RDNA template (C_{T2})

![Diagram of ligation reaction and absorption at 260 nm](image)

| Entry          | Sequence                                    | Expected mass | Observed mass |
|----------------|---------------------------------------------|---------------|---------------|
| Ligand R_{L1} | 5'-UUUUAAAUAOH-3'                           | 2456          | 2457          |
| Ligand R_{L4} | 5'-PO_3{sup 2-}-AUAAUAUU-3'                 | 2582          | 2583          |
| Template C_{T2}| 3'-AAAAUUUAUUUAUUUAUUUAU-5'                 | 4893          | 4893          |
| Ligation product R_{P6} | 5'-UUUUAAAUAUAUAUAUAU-3' | 5021          | 5021          |

**Yield (%)**
- 0 min: 0%
- 30 min: 79%
- 60 min: 79%

**Supplementary Figure 99:** AEC traces of the ligation reaction of RNA ligands (10 µM of each) on DNA-RNA chimeric (R_{T2}) template (10 µM) in presence of T4 RNA ligase 2 at 4 °C. The peak corresponding to the ligation product was observed (Top) which was further confirmed by the MALDI-TOF of the 1 h reaction mixture. Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
Control Reaction in enzymatic ligation reaction: T4 RNA ligase 2 mediated enzymatic ligation reaction between homogeneous RNA ligands in absence of any template

![Diagram of control reaction](image)

### Table: Expected and Observed Masses

| Entry             | Sequence                                  | Expected mass | Observed mass |
|-------------------|-------------------------------------------|---------------|---------------|
| Ligand R<sub>L11</sub> | 5'-UUUUAAAU<sub>OH</sub>-3'                | 2456          | 2458          |
| Ligand R<sub>L4</sub>  | 5'-PO<sub>3</sub>-AUAAUAAU-3'             | 2582          | 2584          |
| Ligation product R<sub>P6</sub> | 5'-UUUUAAUAUAUAUAUAU-3'                  | 5021          | Not observed  |

**Supplementary Figure 100**: AEC traces of the control reaction of RNA ligands (10 µM of each) in absence of any template in presence of T4 RNA ligase 2 at 4 °C. After 2 h, no peak corresponding to the ligation product was observed (Top) which was further confirmed by the MALDI-TOF of the 24 h reaction mixture. Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.
**Enzymatic Ligation reaction.** T4 RNA Ligase2 mediated ligation reaction of Chimeric RDNA ligands on RNA template (R\textsubscript{T2})

\[
\begin{align*}
5'\text{-UUUUAAAU}^\text{OH}.3' & \quad 5'\text{-PO}_3^2\text{-AUAAUAAU}-3' \\
3'\text{-AAAAUUUA} & \quad \text{UAUAAUUA}-5'
\end{align*}
\]

T4 RNA ligase 2, T4 RNA Ligase 2 buffer, 4 °C

**Supplementary Figure 101:** AEC traces of the ligation reaction of DNA-RNA chimeric ligands (10 µM of each) on RNA (R\textsubscript{T2}) template (10 µM) in presence of T4 RNA ligase 2 at 4 °C. Even after 24 h, no peak corresponding to the ligation product was observed (Top) which was further confirmed by the MALDI-TOF of the 24 h reaction mixture. **Gradient:** 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.

| Entry          | Sequence                               | Expected mass | Observed mass |
|----------------|----------------------------------------|---------------|---------------|
| Ligand C\textsubscript{L9}       | 5'-UUUUAAAU^OH-3'                      | 2408          | 2410          |
| Ligand C\textsubscript{L10}      | 5'-PO\textsubscript{3}-AUAAUAAU-3'     | 2502          | 2504          |
| Template R\textsubscript{T2}     | 3'-AAAAUUUAUUAUUUAUUA-5'               | 5021          | 5023          |
| Ligation product C\textsubscript{P5} | 5'-UUUUAAAUAAUAAUAAU-3'              | 4893          | Not observed  |
T4 RNA ligase 2 mediated development of enzymatic replication cycle: Chimeric RDNA template and homogeneous RNA ligands

Supplementary Figure 102: Attempted enzymatic approach of utilizing the 1st ligation product as template for the 2nd ligation reaction using DNA-RNA chimeric C_{T2} template. The approach is analogous to the non-enzymatic one as shown in supplementary Figure 63 & 79.
**T4 RNA ligase 2 mediated development of enzymatic replication cycle**: Chimeric RDNA template and homogeneous RNA ligands

### Entry Sequence

| Ligand R<sub>L11</sub> | 5'-UUUUAAAU<sup>OH</sup>-3' |
|------------------------|-----------------------------|
| Ligand R<sub>L4</sub>  | 5'-PO<sub>2</sub>-AUAAUAUU-3' |
| Ligand R<sub>L12</sub> | 5'-AUUAAUU<sup>OH</sup>-3'    |
| Ligand R<sub>L6</sub>  | 5'-PO<sub>2</sub>-UUUAAA-3'    |
| Template C<sub>T2</sub>| 3'-AAAAUUUUUUUUU-5'          |

| 1<sup>st</sup> Ligation product RP<sub>6</sub> | Yield (%) |
|-------------------------------------------------|-----------|
| 2 h                                               | 79%       |
| 2+2 h                                             | 82%       |
| 2+24 h                                            | 93%       |

| 2<sup>nd</sup> Ligation product RP<sub>7</sub> | Yield (%) |
|-------------------------------------------------|-----------|
| 2+2 h                                            | <5%       |
| 2+24 h                                           | 18%       |

### Supplementary Figure 103: AEC traces of the ligation reaction of the RNA ligands (10 µM of each) on DNA-RNA chimeric (C<sub>T2</sub>) template (10 µM) in presence of T4 RNA ligase 2 at 4 °C with 0.5-unit 1X ligase buffer 2. After 2 h of reaction, ligands, complementary to 1<sup>st</sup> ligation product was added. But, even after 2 h, no peak corresponding to the 2<sup>nd</sup> ligation product was observed (Top). Then 0.5-unit of 1X ligase 2 buffer was further added and after 24 h, a peak corresponding to the 2<sup>nd</sup> ligation product was observed. *Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.*
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