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The role of sugar-backbone heterogeneity and chimeras in the simultaneous emergence of RNA and DNA

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Supplementary Information

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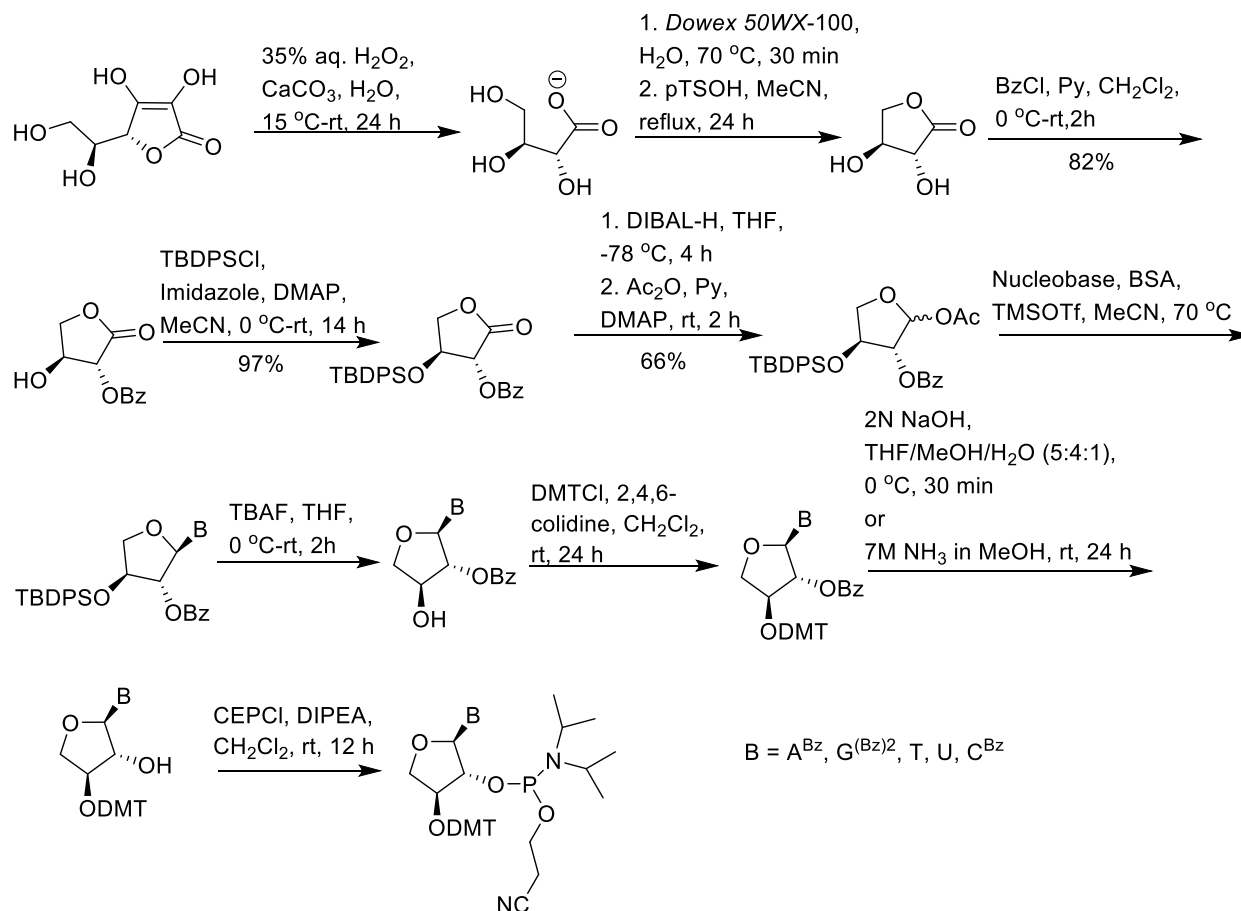
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General Experimental:

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.¹ TNA & TNA-RNA chimeric sequences were synthesized using “Expedite synthesizer” following standard reported protocol.² TNA ligands were utilized from the laboratory inventory made by Wu et al.³ 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 ¹H, ¹³C and ³¹P NMR and are identical to those reported in the literature.^{1,2}



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

Synthesis of oligonucleotide²:

Oligomer synthesis was conducted on a 1 μmol 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'-A ₈ U ₈ -2'(3')	23.6	4777	4775
4	(5')3'-a ₈ U ₈ -3'	24.3	4777	4778
5	5'-AUAUAUAUAUAUAUAU-3'	25.2	4988	4982
6	5'-AUAUAUAUAUAUAUAU-3'	24.4	4958	4957
7	5'-AUAUAUAUAUAUAUAU-3'	21.8	4988	4984
8	(5')3'-aAaAtTtAtAtTaTtA-3'	23.5	4893	4890
9	3'-TtTtAaAtAtAaTtAt-3' (5')	24.2	4893	4891
10	(5')3'-aAaAuUuAuAuUaUuA-3'	24.3	4778	4775
11	3'-UuUuAaAuAuAaUaAu-3'(5')	24.3	4778	4772
12	(5')3'-aaaaTTTAtAttAttA-3'	23.5	4863	4860
13	3'-TTTTaaatAtAAtAAAt-3' (5')	23.5	4923	4920
14	(5')3'-aaagTTTAtAtcGctA-3'	24.5	4865	4863
15	3'-TTTCaaatAtAGcGAt-3' (5')	29.3	4925	4926
16	(5')3'-aaaaTTTA $\underline{T}^{\text{NH}_2}$ -3'	10.9	2727	2728
17	5'-PO ₃ ²⁻ -AttAttA-3'	11.4	2166	2167
18	5'-AAAATTTA $\underline{T}^{\text{NH}_2}$ -3'	12.2	2847	2848
19	5'-PO ₃ ²⁻ -ATTATTA-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) $\geq$ 95%. ^b MALDI was recorded using THAP matrix; a,u,t,g,c = TNA; A,U,T,G,C = RNA; $\underline{T}$ = 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 μ L 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 $^{\circ}$ C per minute. Melting temperatures (T_m) were calculated using the Van't Hoff two-state model and averaged from multiple heating/cooling cycles.

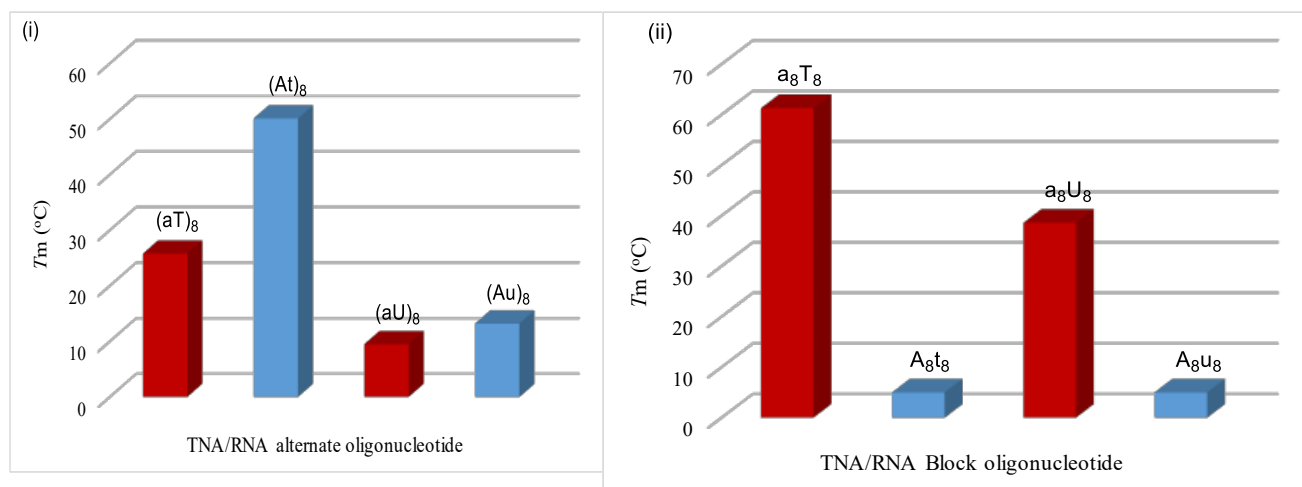
Supplementary Table 2: UV-T_m thermal stability of TNA-RNA chimeric sequences

Entry	Sequence	Conc.(total)	T _m (°C) [*]	ΔT _m (°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 ₈ t ₈ -2'(3')	10 μM	<5 [†]	-40.9
7.	(5')3'-a ₈ T ₈ -3'	10 μM	61.3 [†]	+20.4
8.	5'-A ₈ u ₈ -2'(3')	10 μM	<5	-40.9
9.	(5')3-a ₈ U 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'-AUAUAUAUaUAUAUAU-3'	10 μM	46.4	-7.2
13.	5'-AAAATTTAt A TTATTA-3'+ 3'-TTTAAATATAATAAT-5'	(2+2) μM	43.8 ^{**}	-5.3
14.	5'-AAAAT T t At ATTAT TA-3'+ 3'-T T TTAAATATAATAAT-5'	(2+2) μM	33.5 ^{**}	-15.4
15.	5'-AAAA t t t A t At t At t A-3'+ 3'-TT T TAAATATAATAAT-5'	(2+2) μM	<10 ^{**}	-
16.	(5')3'-aAaAtTtAtAtTaTtA-3'	10 μM	-	-
17.	(5')3'-tAaTaAtAtAaAtTtT-3'	10 μM	-	-
18.	(5')3'-aAaAtT tAtAtT aTtA-3'+ 3'-T tT tAaAtAtAaTaAt-3' (5')	(10+10) μM	-	-

Entry	Sequence	Conc.(total)	T _m (oC)*	ΔT _m (°C)
19	(5')3'-aAaAuUuAuAuUaUuA-3' 3'-UuUuAaAuAuAaUaAu-3'(5')	(5+5) μM	-	-
20	(5')3'-aAaAtT t AtA tT aTtA-3'+ 3'- <u>TTTTAAATATAATAAT</u> -5'	(2+2) μM	<5	-
21	5'- <u>AAAATTTATATTATTA</u> -3'+ 3'-TtT t AaAtA tAa TaAt-3' (5')	(2+2) μM	<5	-
RNA series for comparison				
22	5'-(AU) ₈ -3'	10 μM	53.6	
23	5'-A ₈ U ₈ -3'	10 μM	40.9	
14	5'-AAAATTTATATTATTA-3' + 3'-TTTTAAATATAATAAT-5'	(2+2) μM	48.9** 59.5**	

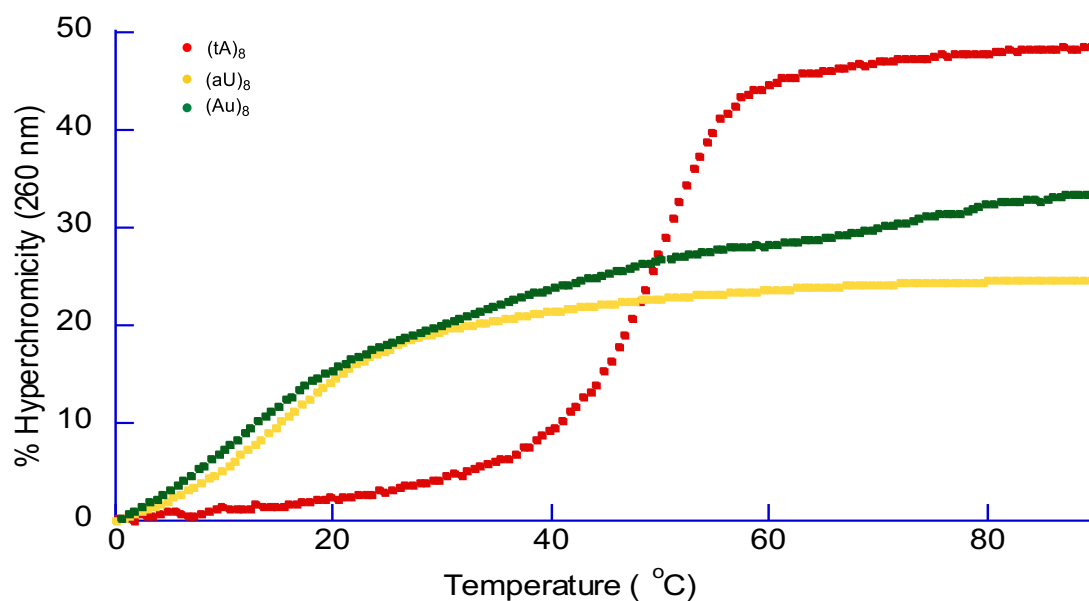
* 1M NaCl, 10 mM Na₂HPO₄, 100 μM EDTA pH 7.2 buffer; † Reference 2; ** Reference 4; # ΔT_m is w.r.t. corresponding RNA sequences; A,U,T = RNA, a,u,t = TNA, A,T = DNA.

Strong-Weak base pairing trend in TNA-RNA chimeras

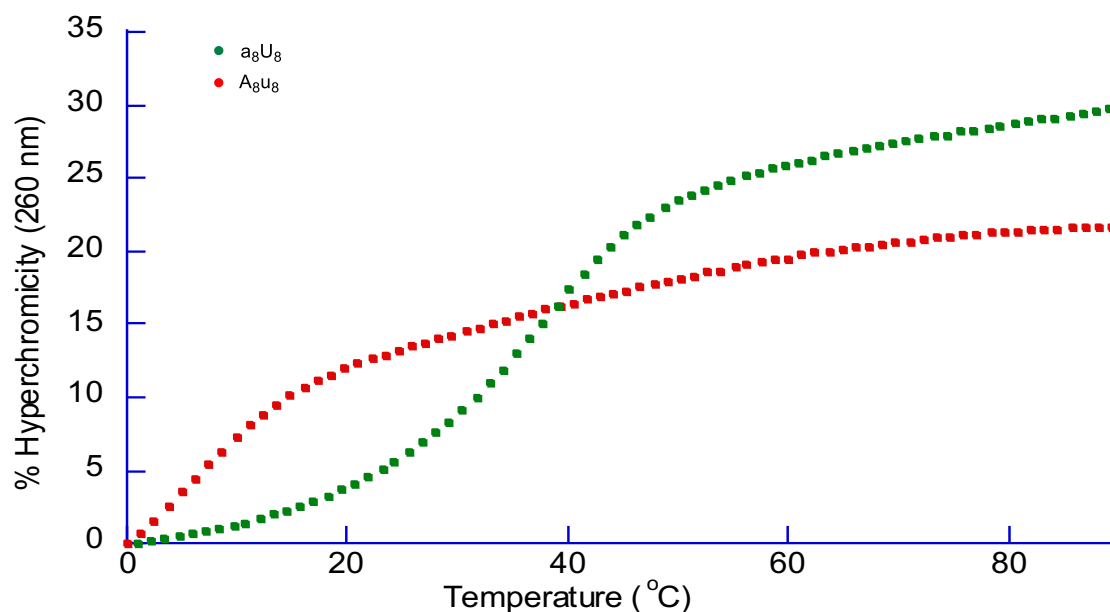


Supplementary Figure 2. A dichotomous thermal stability behavior of self-complementary hexadecameric TNA-RNA chimeric duplexes. Figure (i), left side shows that in an alternate TNA-RNA chimeric sequence TNA-pyrimidine and RNA-purine gave stronger duplexes (Table 2, Entry 1-5) while in Figure ii (right 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.

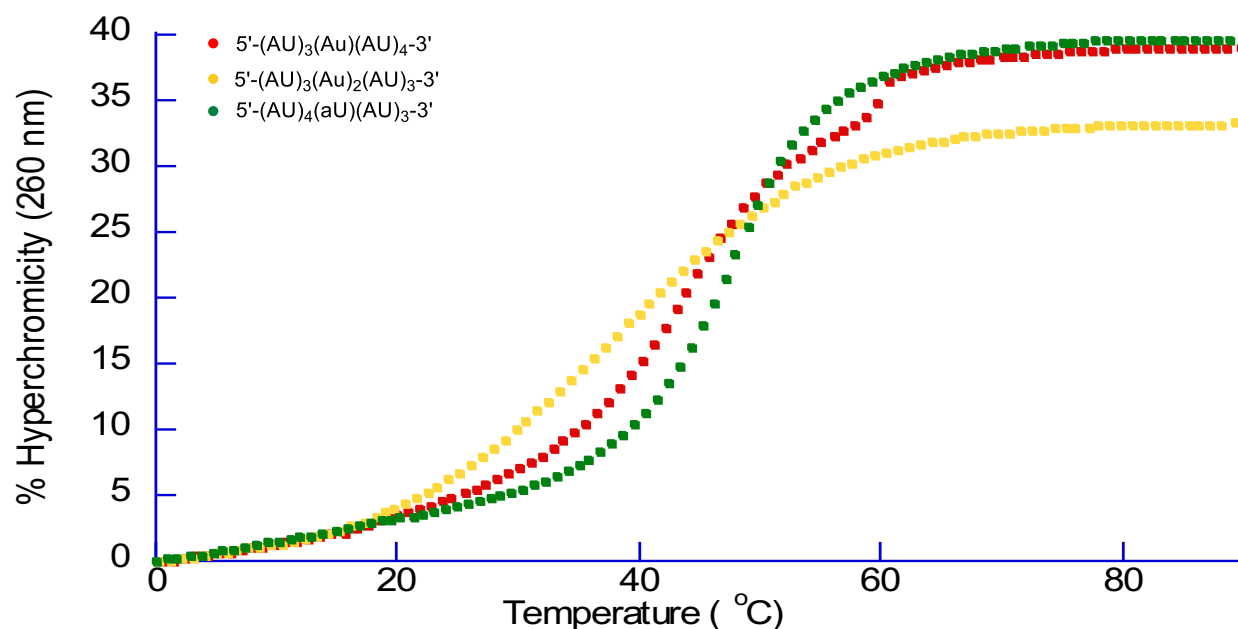
UV-melting curves



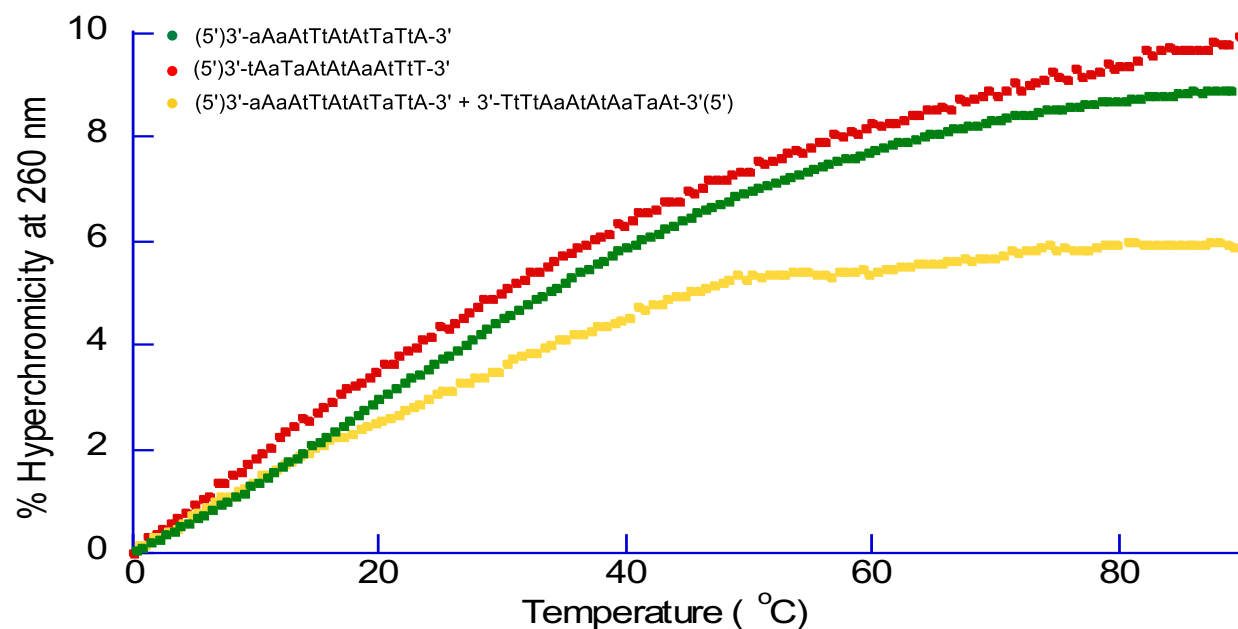
Supplementary Figure 3. Melting curves for self-complementary alternate TNA-RNA chimeric sequences (Supplementary Table 2, Entry 2,4-5) in 10 mM Na₂HPO₄, and 1 M NaCl, 100 μM EDTA at pH 7.2; a,t,u = TNA and A,T,U = RNA.



Supplementary Figure 4. Melting curves for self-complementary alternate TNA-RNA chimeric sequences (Supplementary Table 2, Entry 8-9); in 10 mM Na₂HPO₄, 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 Na₂HPO₄, 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 Na₂HPO₄, 1 M NaCl, 100 μM EDTA at pH 7.2; a,u = TNA and A,U = RNA.

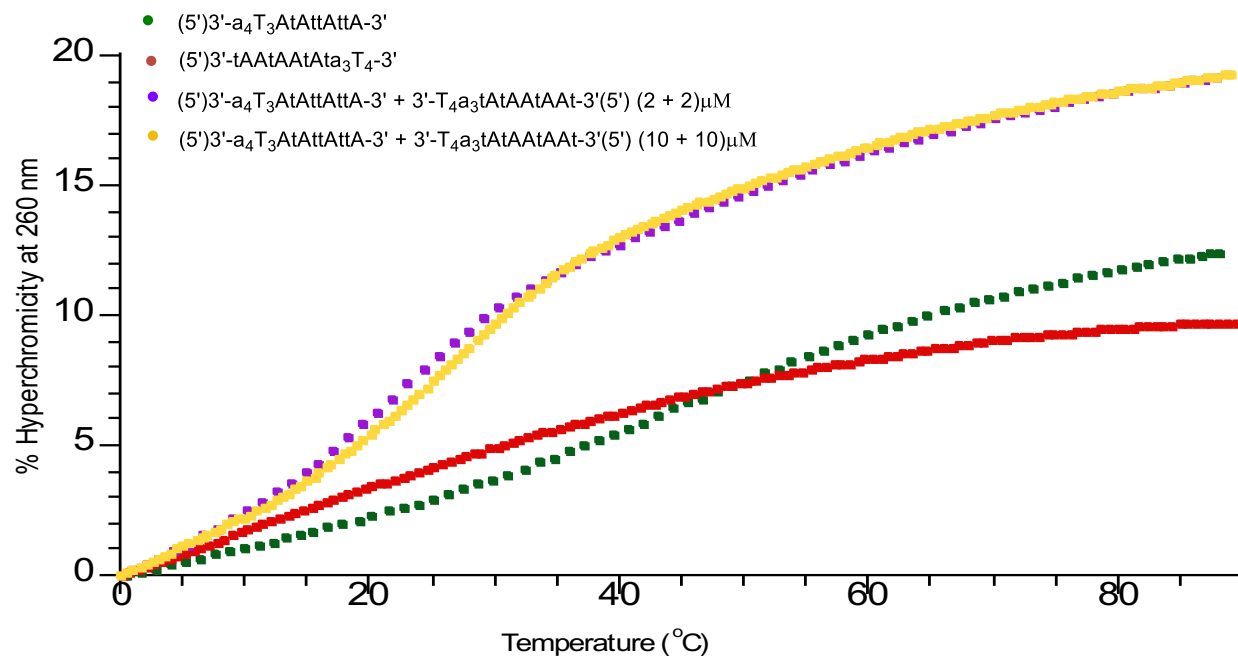
Supplementary Table 3: Duplex thermal stability of TNA/RNA chimeric sequences and it's complementary strands

Entry	Sequence	Description	$T_m(^{\circ}\text{C})^a$ $\approx (2+2) \mu\text{M}$	$\Delta T_m(^{\circ}\text{C})^{\#}$
1	(5')3'-aaaaTTTAtAttAttA-3'	TRNA	-	-
2	(5')3'-tAAtAAtAtaaaTTTT-3'	TRNA	-	-
3	(5')3'-aaaaTTTAtA t tA t tA-3'+ 3'-TTTTaaatAtAAAtAAAt-3' (5')	TRNA TRNA	<20* <20''*	> -39.5 > -39.5
4	(5')3'-aaaa TTTAtA t t At t A-3'+ 3'- <u>TTTTAAATATAATAAT</u> -5'	TRNA DNA	20.2	-39.3
5	<u>5'-AAAATTTATATTATTA-3'</u> + 3'-TTTTa aat AtAAAtAAAt-3' (5')	DNA TRNA	25	-34.5
6	5'-AAAATTTATATTATTA-3'+ 3'-T TT T aa a t At AAAt AAAt-3' (5')	RNA TRNA	40.5	-19.0
7	5'-AAAATTTA <u>I</u> (NH)ATTATTA-3' + 3'-TTT T a aat A—— t AAAt AAAt-3' (5')	R(NH)NA TRNA	42	-17.5
8	5'-AAAATT TA <u>I</u> (NH)AT TAT TA-3' + 3'-UUUUAAAUA——UAAUAAU-5'	R(NH)NA RNA	53.9 ^b	-6.6
9	3'-a a aa tt ta ta t ta t ta-2'+ 3'-TTTTaaatAtAAAtAAAt-3' (5')	TNA TRNA	38.3	-21.2
10	(5')3'-a a aaT T TAt A t t A t t A-3'+ 3'-TTTTAAATATAATAAT-5'	TRNA RNA	35.5	-24.0
11	(5')3'-a a a aT TTA t A t t A t t A-3'+ 3'-UUUUAAAUAUAAUAAU-5'	TRNA RNA	31.6	-27.9
12	5'-AAAAUUUAUUAUUAUUA-3'+ 3'-T TT T a a a t A t AAAt AA t-3' (5')	RNA TRNA	25.1	-34.4

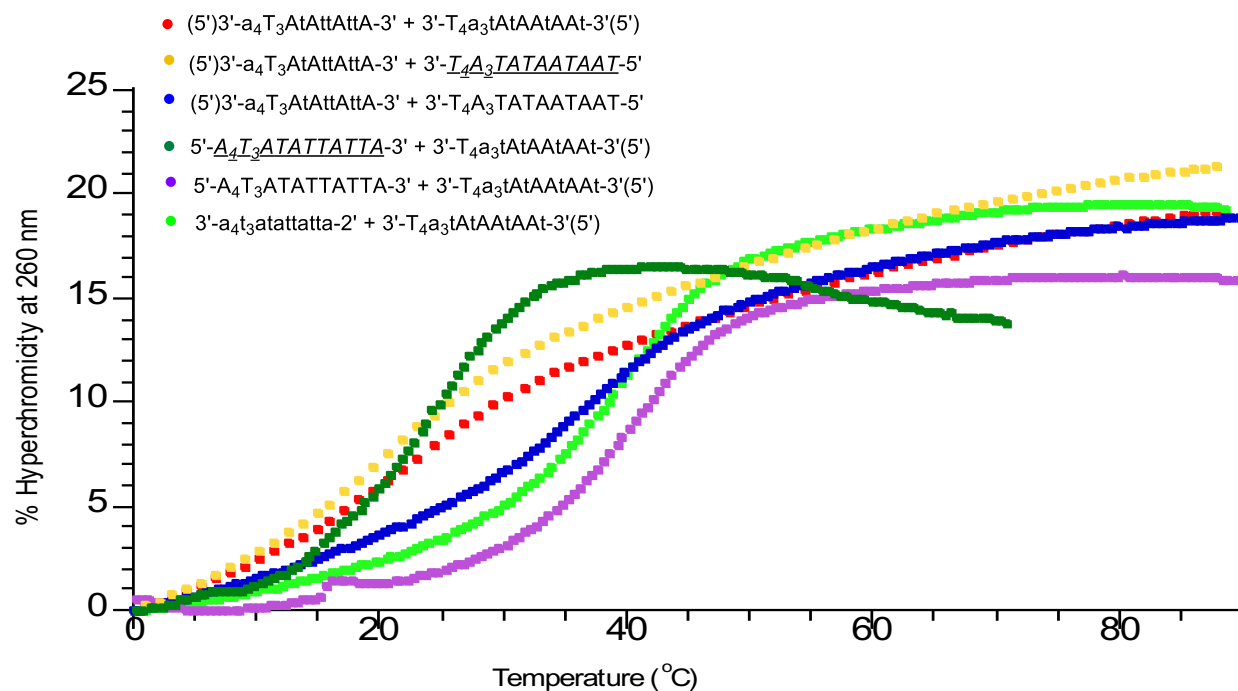
Entry	Sequence	Description	$T_m(^{\circ}\text{C})^a$ $\approx (2+2) \mu\text{M}$	$\Delta T_m(^{\circ}\text{C})^{\#}$
13	(5')3'-a a agTTTAtAt cGc tA-3'+ 3'-TTTCa a atAtAGcGAt-3' (5')	TRNA TRNA	<30*	> - 35.5
14	(5')3'-a a a gTTT A t A t c Gc t A-3'+ 3'-UUUCAAUAUAGCGAU-5'	TRNA RNA	<45*	> - 25.5
15	5'-AAAGUUUAUAUCGCUA-3'+ 3'-T TT Ca a a t A t A Gc GAt-3' (5')	RNA TRNA	52.5	-13
RNA series for comparison				
16	5'-AAAATTTATATTATTA-3' + 3'-TTTTAAATATAATAAT-5'	RNA RNA	59.5 48.9 ^{b§}	
17	5'-AAAGUUUAUAUCGCUA-3'+ 3'-UUUCAAUAUAGCGAU-5'	RNA RNA	65.5	

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; * No clear sigmoidal melting curve was observed; [†] $\approx (10+10) \mu\text{M}$; [#] ΔT_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.

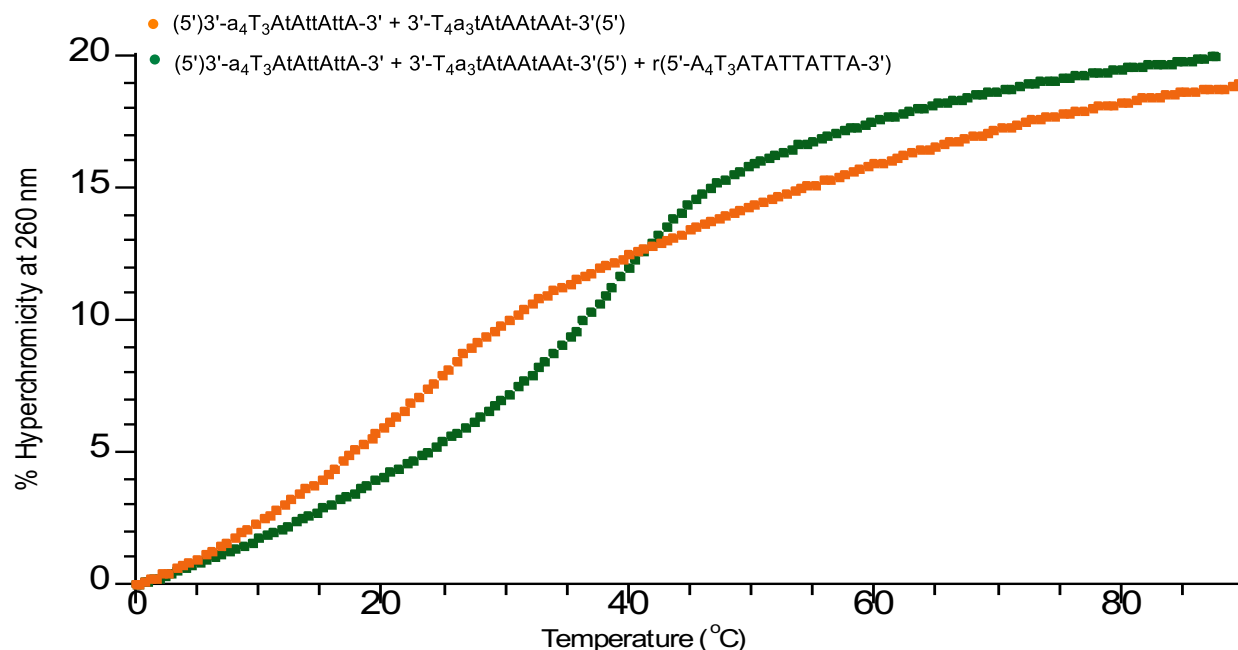
UV melting curves



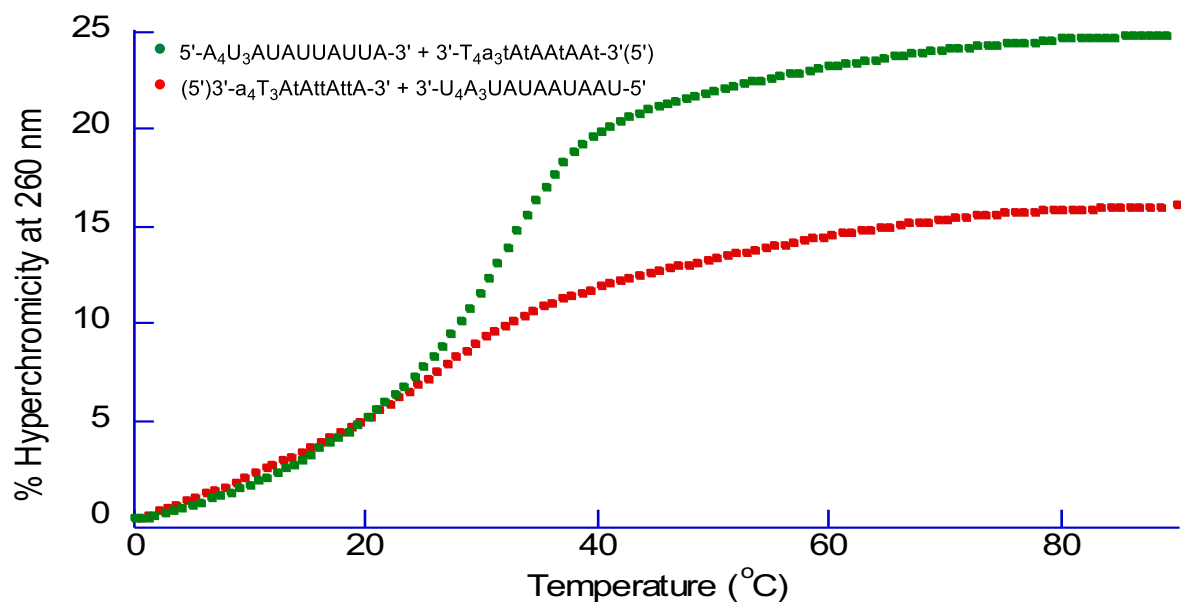
Supplementary Figure 7. Melting curves of TNA-RNA chimeric sequences (single strands and duplexes) (Supplementary Table 3, Entry 1-3) in 10 mM Na₂HPO₄, 1 M NaCl, 100 μ M EDTA at pH 7.2 buffer; a,t = TNA and A,T = RNA.



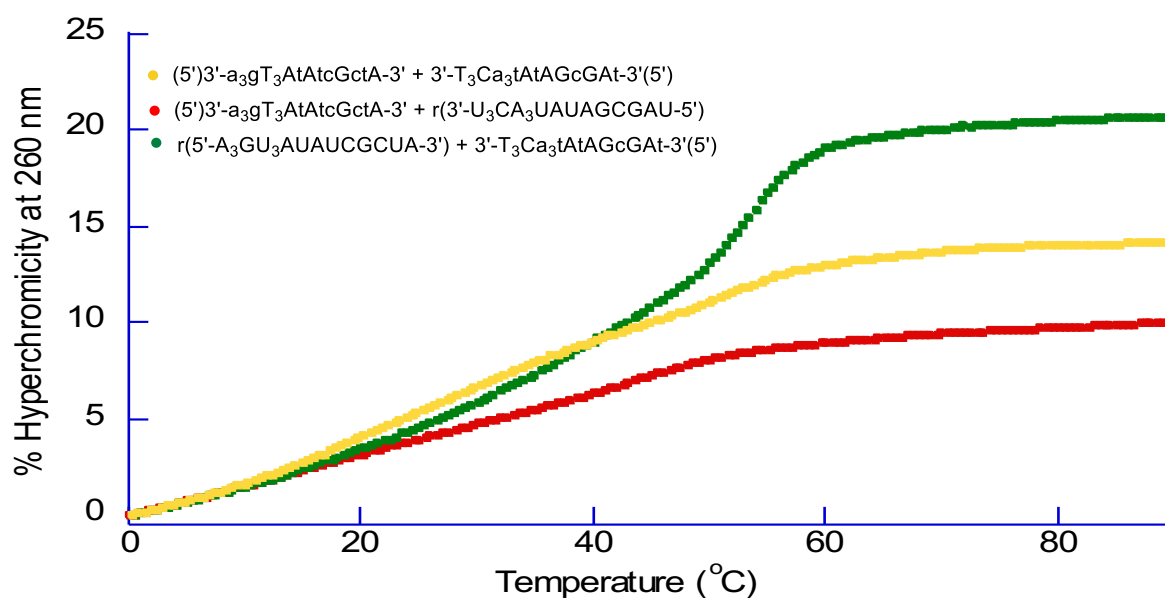
Supplementary Figure 8. Melting curves of TNA-RNA chimeric sequences and their complementary heterogeneous and homogeneous duplex strands in 10 mM Na₂HPO₄, 1 M NaCl, 100 μM EDTA at pH 7.2 buffer. It shows that the TNA-RNA chimeric strand prefers complementary homogeneous strands over its TNA-RNA heterogeneous counterpart (Supplementary Table 3, Entry 3-6,9-10). a,t = TNA, A,T = RNA; A,T = DNA.



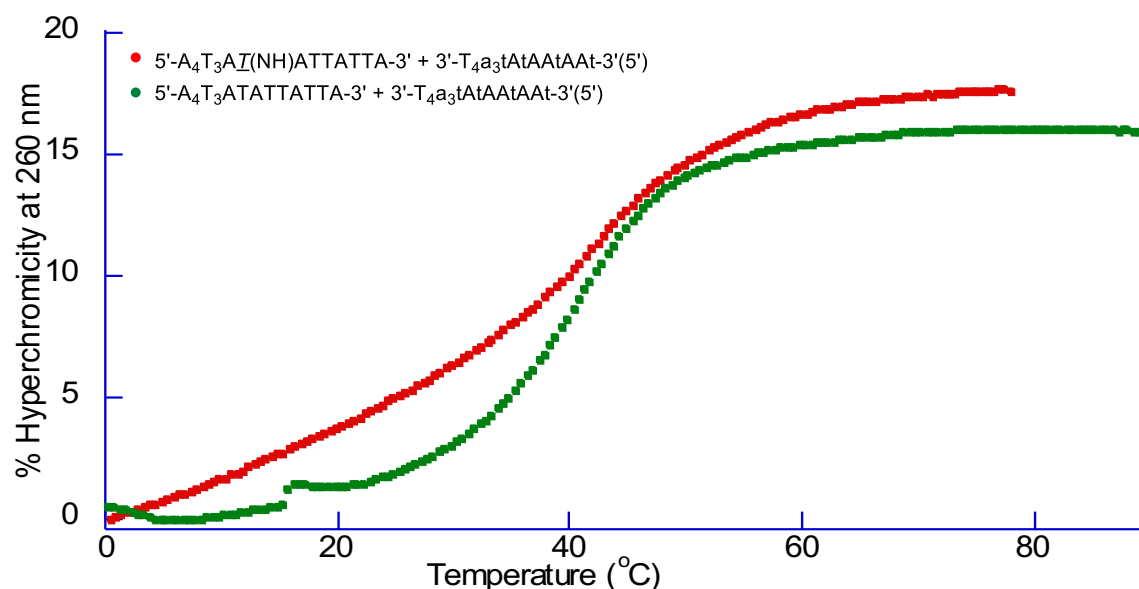
Supplementary Figure 9. Melting curves of TNA-RNA chimeric sequences indicates the preference of heterogeneous TNA-RNA chimeric sequence for the homogeneous RNA sequence (green) over the heterogeneous TNA-RNA sequence (orange) in 10 mM Na₂HPO₄, 1 M NaCl, 100 μM EDTA at pH 7.2 buffer. The orange line indicates the UV-T_m curve of complementary TNA-RNA chimeric sequence and the green line indicates the UV-T_m 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.



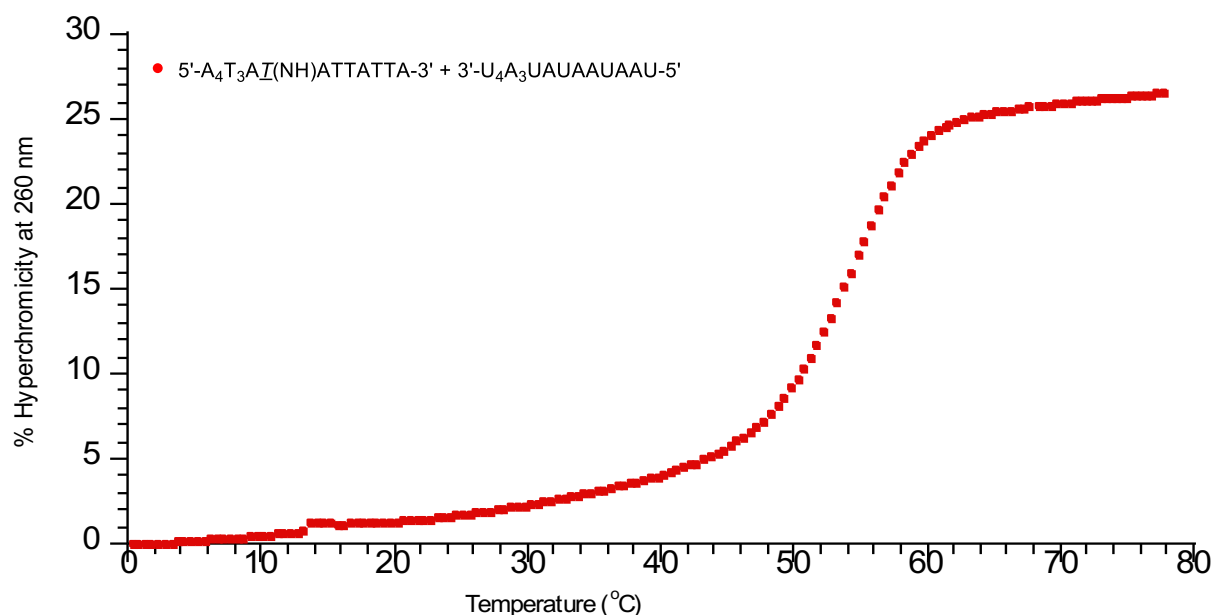
Supplementary Figure 10. Melting curves comparison of the duplexes formed from the two possible combinations of TNA/RNA chimeric sequences with their corresponding complementary homogeneous RNA (A,U) sequences (Supplementary Table 3, Entry 11-12) in 10 mM Na₂HPO₄, 1 M NaCl, 100 μM EDTA at pH 7.2 buffer; 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 Na₂HPO₄, 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 Na₂HPO₄, 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 Na₂HPO₄, 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 μ L 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 μ L of reaction mixture was pipetted out and diluted to 450 μ L 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 $\pm$ 5%.

General procedure for the step-wise dilution experiment. Properly measured aliquots of template (10 μ M) and ligands R_{L3} , R_{L4} , R_{L5} , R_{L6} (20 μ M of each) in a microcentrifuge tube at 30 °C was lyophilized to dryness. To the dried mixture, 6 μ L 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 μ L of the reaction mixture was pipetted off for analysis (see below) and the remaining mixture (5 μ L) was allowed to continue at 4 °C. After 24 h, 1 μ L 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 μ M of each and NO TEMPLATE) were lyophilized and 1 μ L of freshly prepared ligation buffer was added and the ligands mixture was transferred to the remaining volume (4 μ L) of 24 h reaction mixture (final volume became 1+4 = 5 μ L). Therefore, the overall template concentration was decreased but the ligands' concentrations remain the same (20 μ M) throughout the reaction. The procedure of removal of 1 μ L 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 $\pm$ 5%.

AEC analysis: The 1 μ L of the reaction mixture was diluted to 0.5 mL with nuclease free water and 1 μ L of 10 μ M dT₂₄ was added to it (as the external standard), and the mixture was filtered through Millex-GV PVDF filter (4 mm, 0.22 μ m). 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 μ M) and ligands (10 μ M) were lyophilized to dryness at 30 °C in a SpeedVac evaporator. To that dried mixture, 2 μ L of T4 DNA ligase buffer (10X), 17 μ L 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 μ L of 20-unit T4 DNA Ligase (New England Biosciences) was added (final ligase concentration 1 unit/ μ L) and the reaction was allowed to continue at 4 °C. At different time interval, 1 μ L 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 μ M) and ligands (10 μ M) were lyophilized to dryness at 30 °C in a SpeedVac evaporator. In to that, 2 μ L of T4 RNA ligase 2 buffer (10X), 17 μ L 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 μ L of 10-unit T4 DNA Ligase (from New England Biosciences) was added (final ligase concentration became 0.5 unit/ μ L) and the reaction was allowed to continue at 4 °C. At different time interval, 1 μ L 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 $\{(\text{area of ligation product peak}/\text{area of template peak}) \times 100\}$ 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

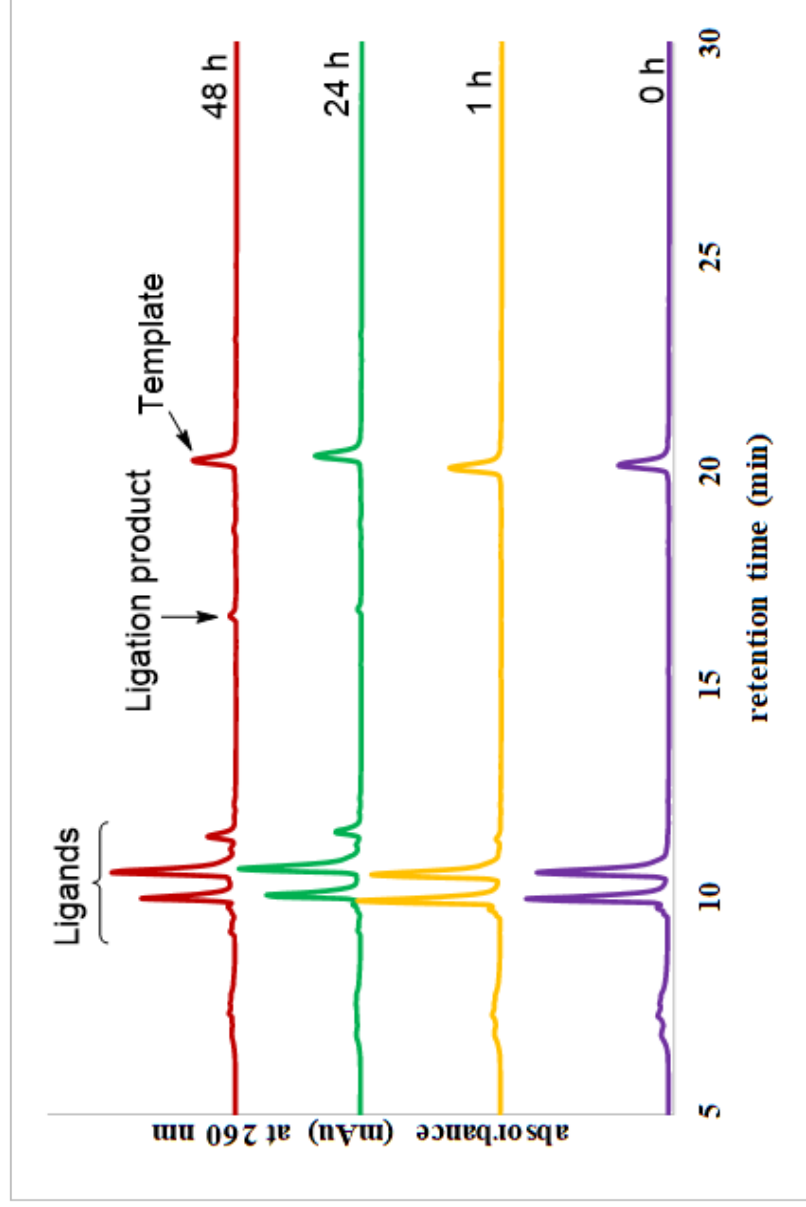
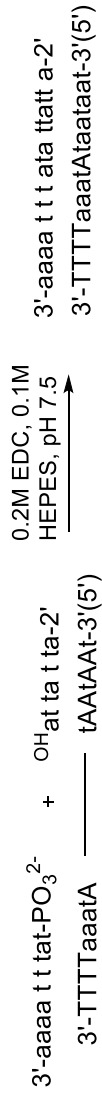
dilution experiment the % yields were calculated w.r.t. an externally added standard oligo (dT₂₄) just before the injection in to the AEC. Reactions were run in triplicates and the yield calculation was within $\pm 5\%$.

Buffers for AEC analysis of ligation reaction: Buffer A: 10 mM Na₂HPO₄, pH 11. Buffer B: 10 mM Na₂HPO₄, 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 μ L micropipette and then washed consecutively with acetonitrile (3 X 4 μ L) and 0.1M TEAAc buffer (3 X 4 μ L). 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 μ L 1:1 acetonitrile/H₂O. Then 1 μ L of the desalted reaction mixture (in 1:1 MeCN/H₂O) was spotted on the MALDI plate with 1 μ L of THAP matrix and analyzed.

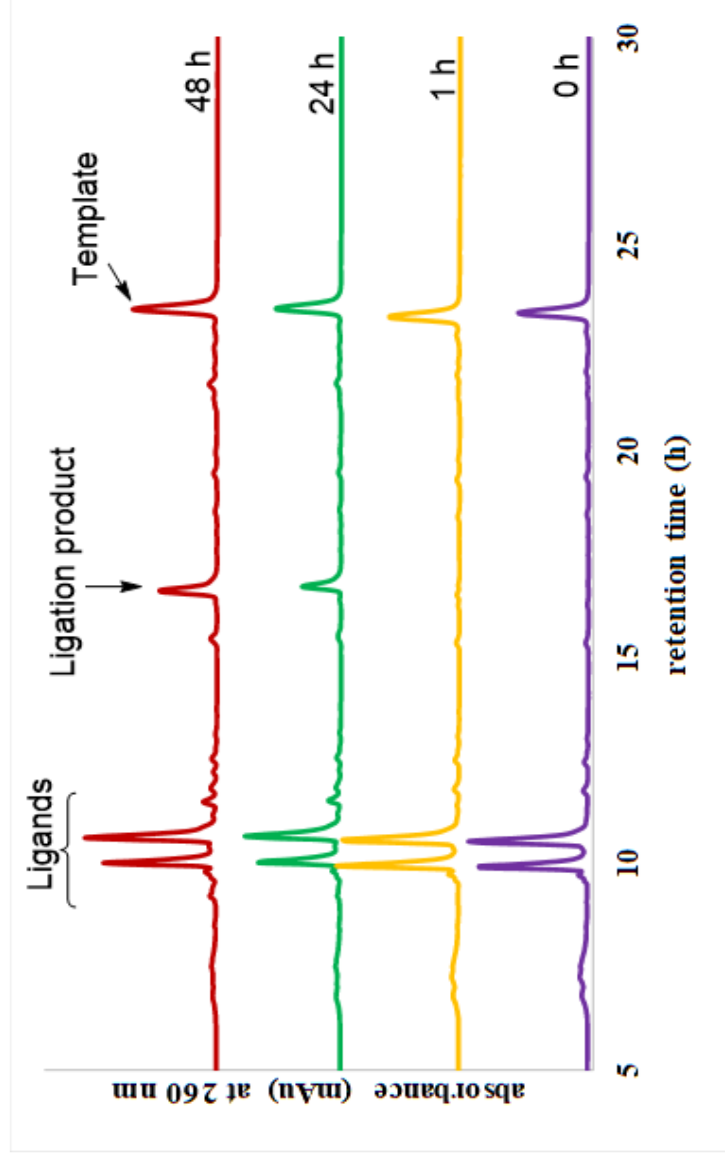
Ligation reaction: TNA ligands (T_{L1} , T_{L2}) on an chimeric tRNA template (C_{T1})



Entry	Sequence	Expected mass	Observed mass
Ligand T_{L1}	3'-aaaaatttat- PO_3^{2-} -2'	2674	2674
Ligand T_{L2}	3'-OH ⁺ attatta-2'	1996	1996
Template C_{T1}	3'-TTTTTaaatAtAAAtAAAt-3' (5')	4923	4920
Ligation product T_{P1}	3'-aaaaatttatattatta-2'	4652	NOT
Yield (%)	0% 7% 48 h	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.*

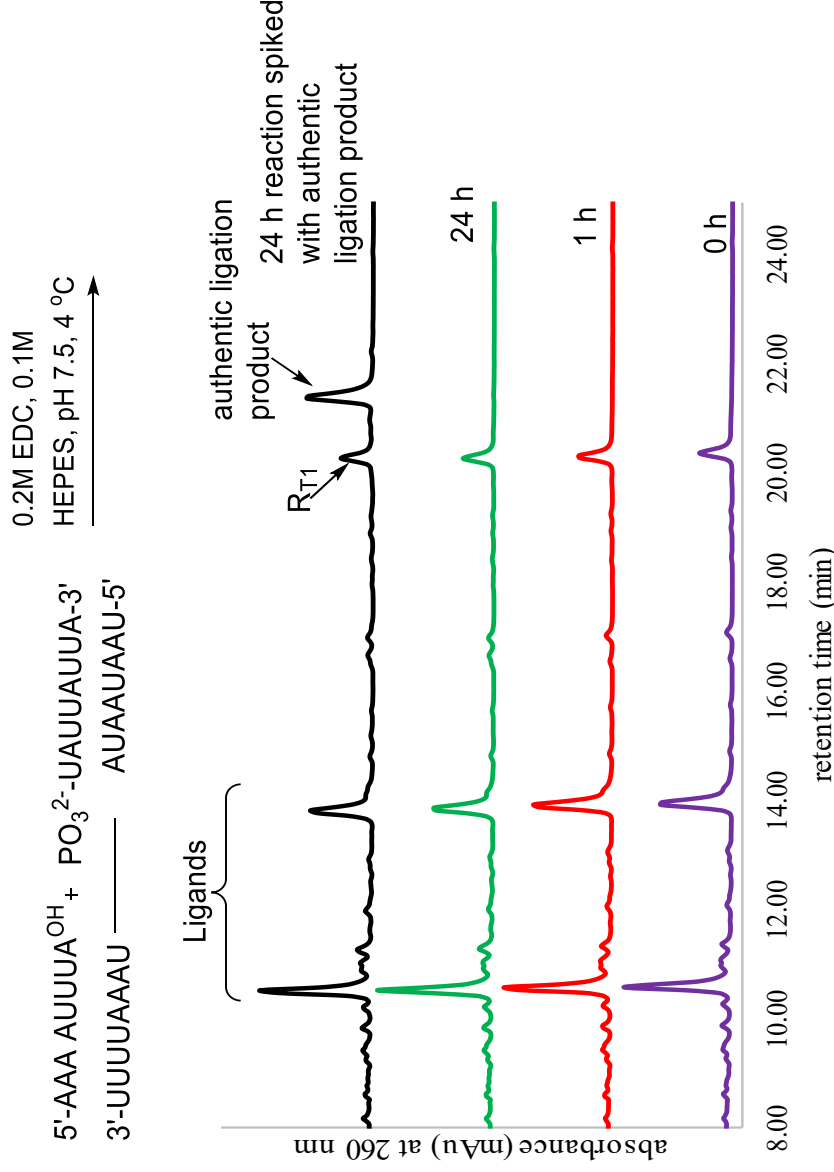
Ligation reaction: TNA ligands (T_{L1}, T_{L2}) on an RNA template (R_{T1})



Entry	Sequence	Expected mass	Observed mass
Ligand T _{L1}	3'-aaaatttat-PO ₃ ²⁻ -2'	2674	2673
Ligand T _{L2}	3'-OHattatta-2'	1996	1996
Template R _{T1}	3'-UUUUUUUUUAUAAUAAU-5'	5021	5016
Ligation product T _{P1}	3'-aaaatttatattatta-2'	4652	4649
Yield (%)	1 h 24 h		
	0% 50% 64%		

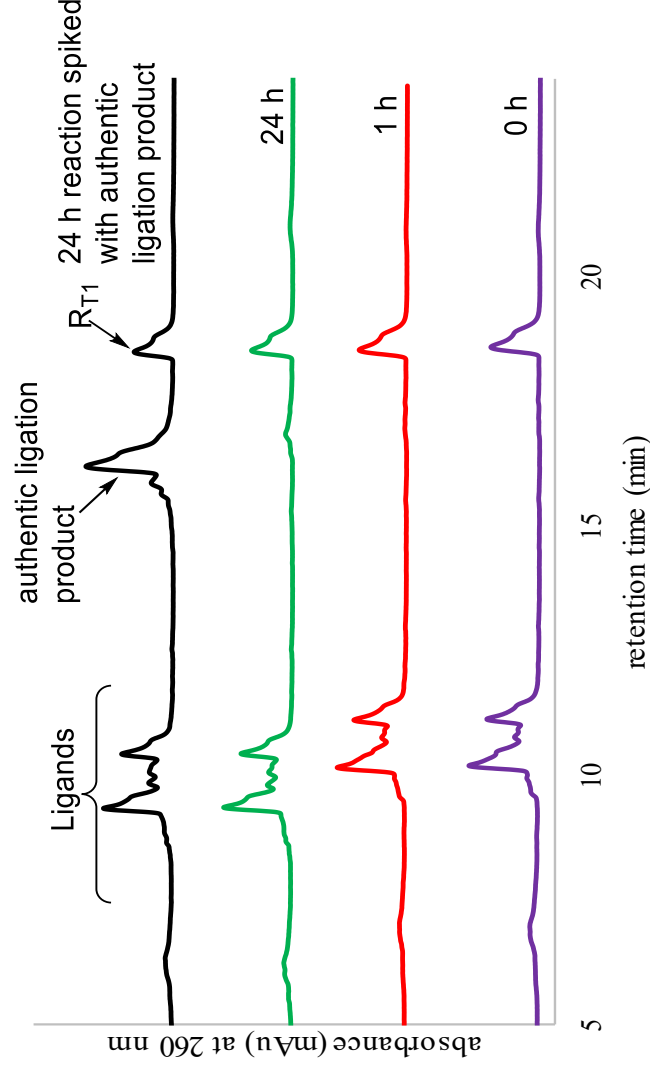
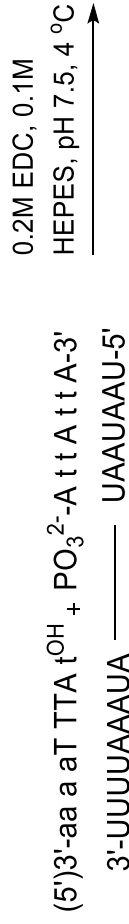
Supplementary Figure 15. AEC trace of the ligation reaction of TNA with 2'-OH ligands (0.2 mM of each) on RNA template (0.1 mM) in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate the appearance of ligation product peak at lower retention time than the template peak. The rate of reaction is very slow compared to the homogeneous TNA ligands with 2'-NH₂. 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'-PO₃²⁻ and 3'-OH) on RNA template (R_{T1})



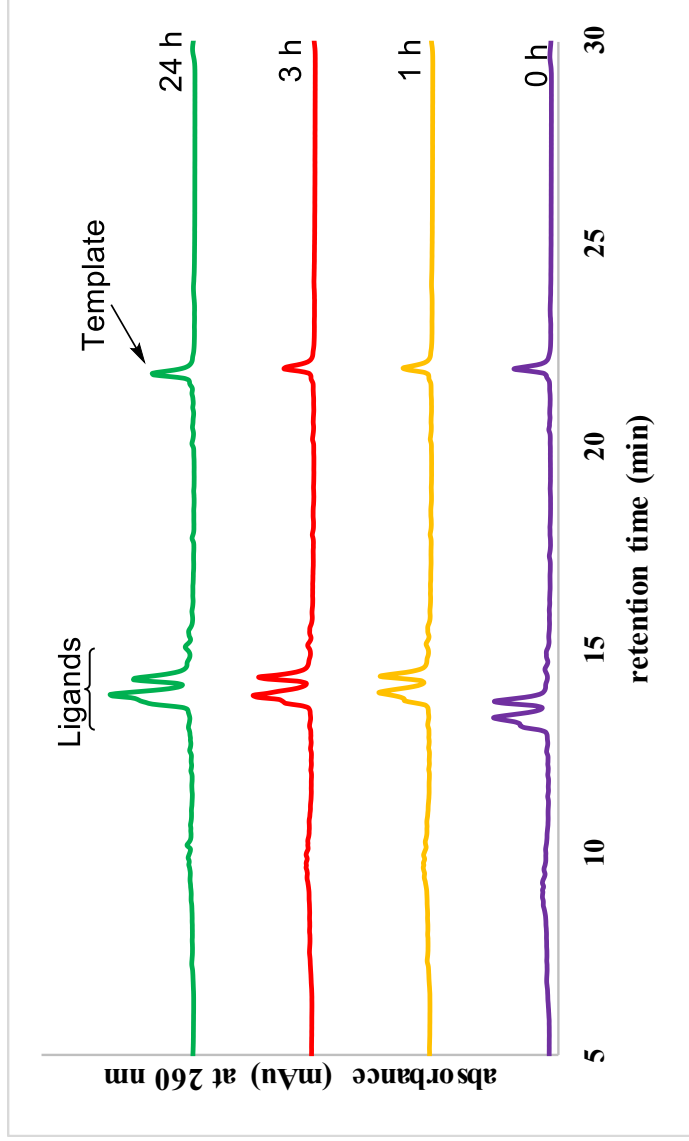
Supplementary Figure 16. AEC trace of the ligation reaction of canonical RNA ligands (0.2 mM of each) on RNA template (0.1 mM) in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate that even after 24 h of reaction no ligation product peak appeared which was confirmed by spiking with authentic 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'-PO₃²⁻ and 2'-OH) on RNA template (R_{T1})



Supplementary Figure 17. AEC trace of the ligation reaction of TRNA chimeric ligands (5'-PO₃²⁻ 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.*

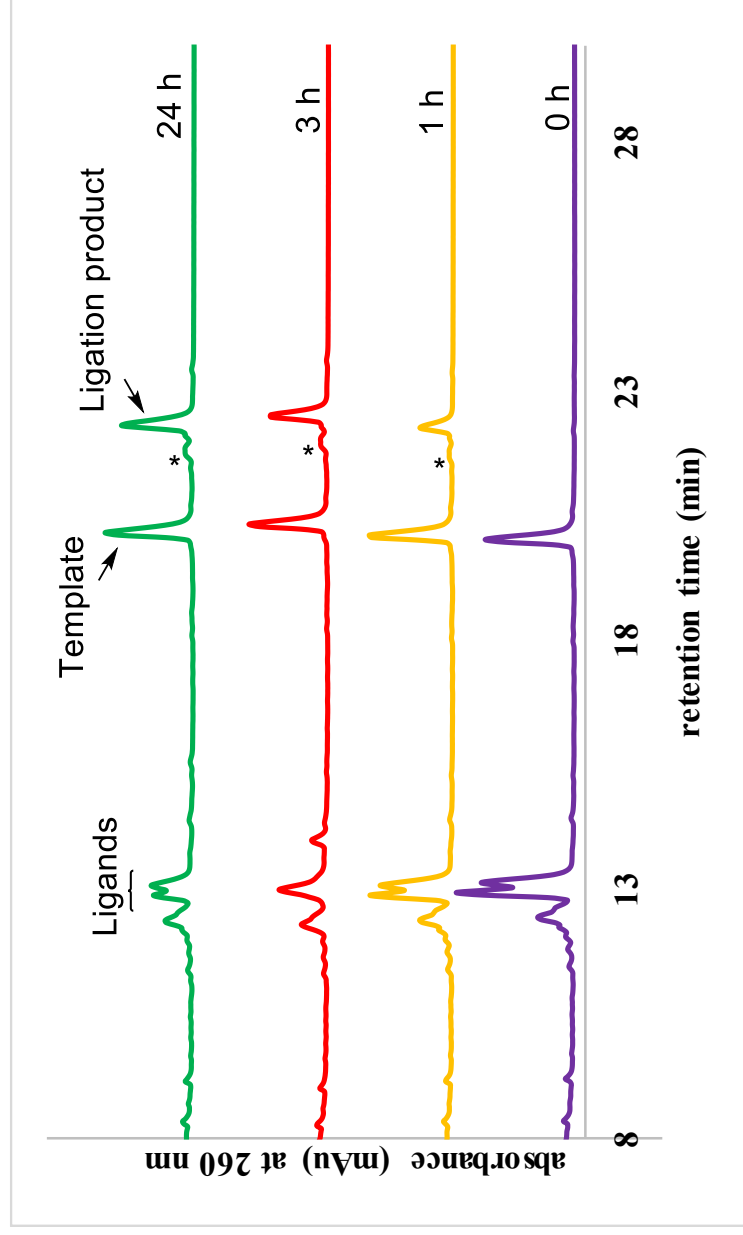
Ligation reaction: TRNA chimeric ligands (C_{L1}, C_{L2}) on chimeric TRNA template (C_{T1})



Entry	Sequence	Expected mass	Observed mass
Ligand C_{L1}	(5')3'-aaaaTTTA $\underline{T}^{\text{NH}_2}$ -3'	2727	2729
Ligand C_{L2}	5'-PO ₃ ²⁻ -AttAttA-3'	2166	2168
Template C_{T1}	3'-TTTTTaaatAtAAAtAAAt-3'(5')	4923	4925
Ligation product C_{PI}	(5')3'-aaaaTTTA $\underline{T}(\text{NH})$ AttAttA-3'	4876	4876 (Not observed in AEC)

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.*

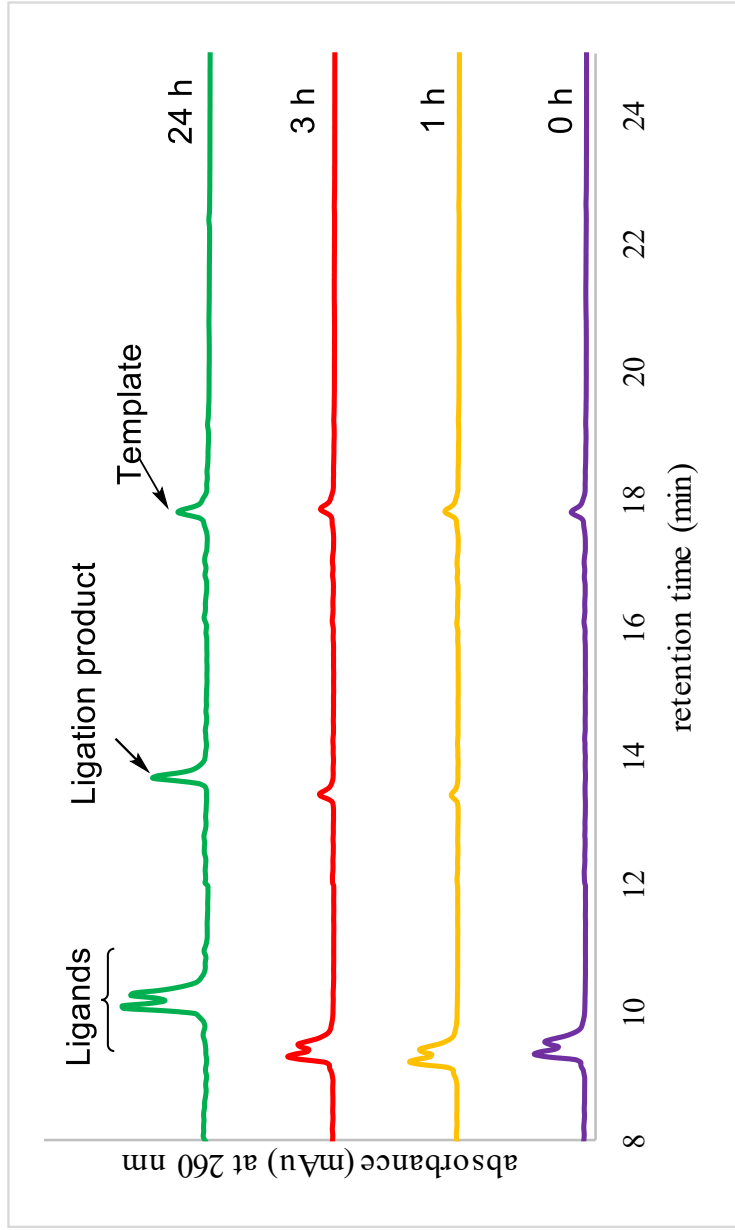
Ligation reaction: RNA ligands (R_{L1}, R_{L2}) on chimeric tRNA template (C_{T1})



Entry	Sequence	Expected mass	Observed mass
Ligand R_{L1}	5'-AAAAATTTA $\underline{\text{I}}^{\text{NH}_2}$ -3'	2847	2852
Ligand R_{L2}	5'-PO ₃ ²⁻ -ATTATTA-3'	2286	2289
Template C_{T1}	3'-TTTTTaaatAAtAAatAAat-3'(5')	4923	4928
Ligation product R_{P1}	5'-AAAAATTTA $\underline{\text{I}}^{\text{(NH)}}\text{ATTATTA-3'}$	5116	5122
Yield (%)	1 h 3 h 24 h		
	23% 51% 102%		

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). *Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.* * unassigned background peak.

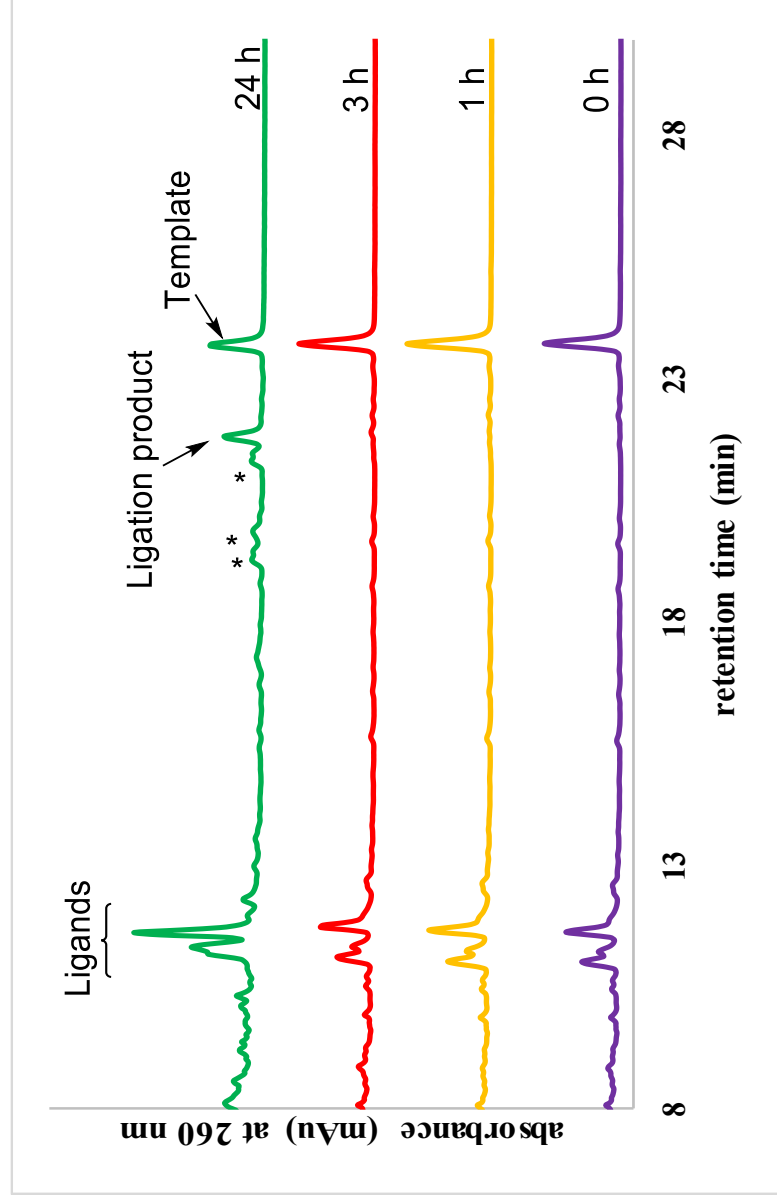
Ligation reaction: TNA ligands (T_{L1}, T_{L3}) on chimeric RTNA template (C_{T1})



Entry	Sequence	Expected mass	Observed mass
Ligand T_{L1}	3'-aaaaatttat-PO ₃ ²⁻ -2'	2674	2676
Ligand T_{L3}	3'-NH ₂ attatta-2'	1995	1997
Template C_{T1}	3'-TTTTaaatAtAAAtAAt-3'(5')	4923	4924
Ligation product T_{P2}	3'-aaaaatttat(NH)attatta-2'	4651	4653
Yield (%)	1 h 3 h 24 h		
	51% 76% 99%		

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.*

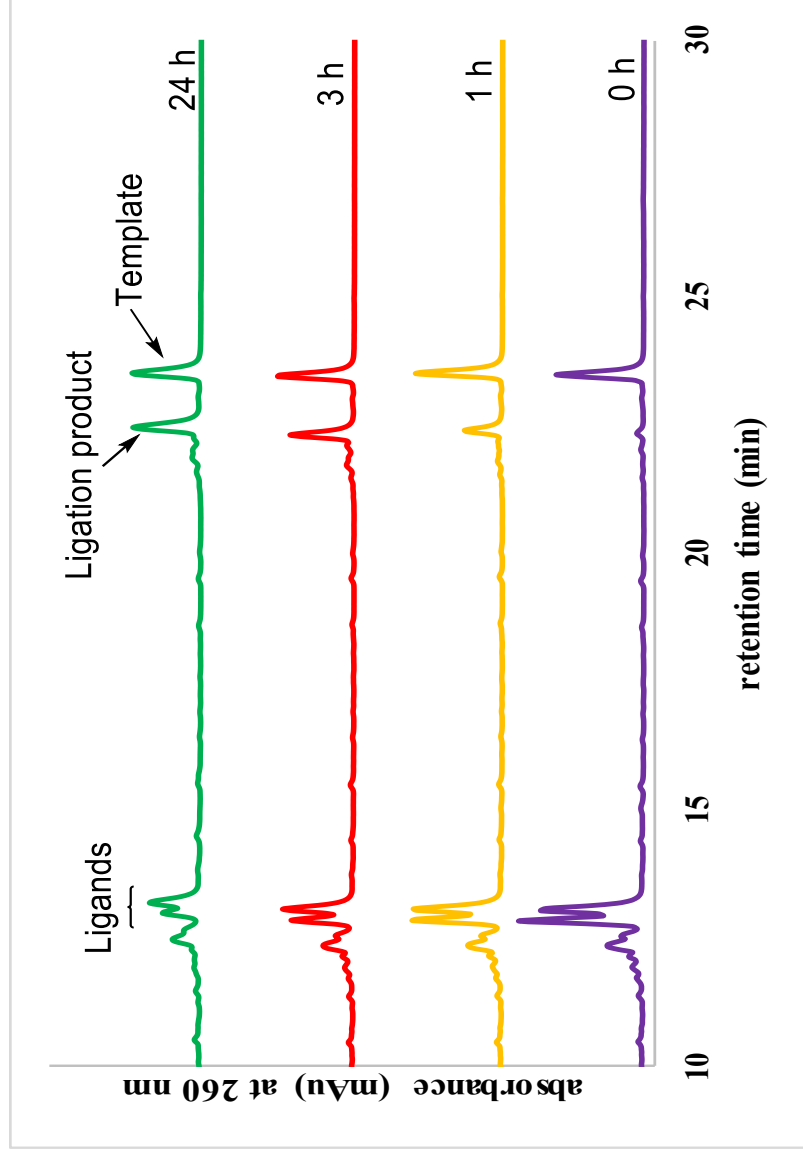
Ligation reaction: tRNA chimeric ligands (C_{L1}, C_{L2}) on RNA template (R_{T1})



Entry	Sequence	Expected mass	Observed mass
Ligand C _{LI}	(5')3'-aaaaTTTA $\overline{T}$ NH ₂ -3'	2727	2728
Ligand C _{LI2}	5'-PO ₃ ²⁻ AttAttA-3'	2166	2167
Template R _{TI}	3'-UUUUAAAUAUAUAAU-5'	5021	5021
Ligation product C _{PI}	(5')3'-aaaaTTTA $\overline{T}$ (NH)AttAttA-3'	4876	4876
Yield (%)	1 h 3 h 24 h		
	0% 5% 71%		

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.

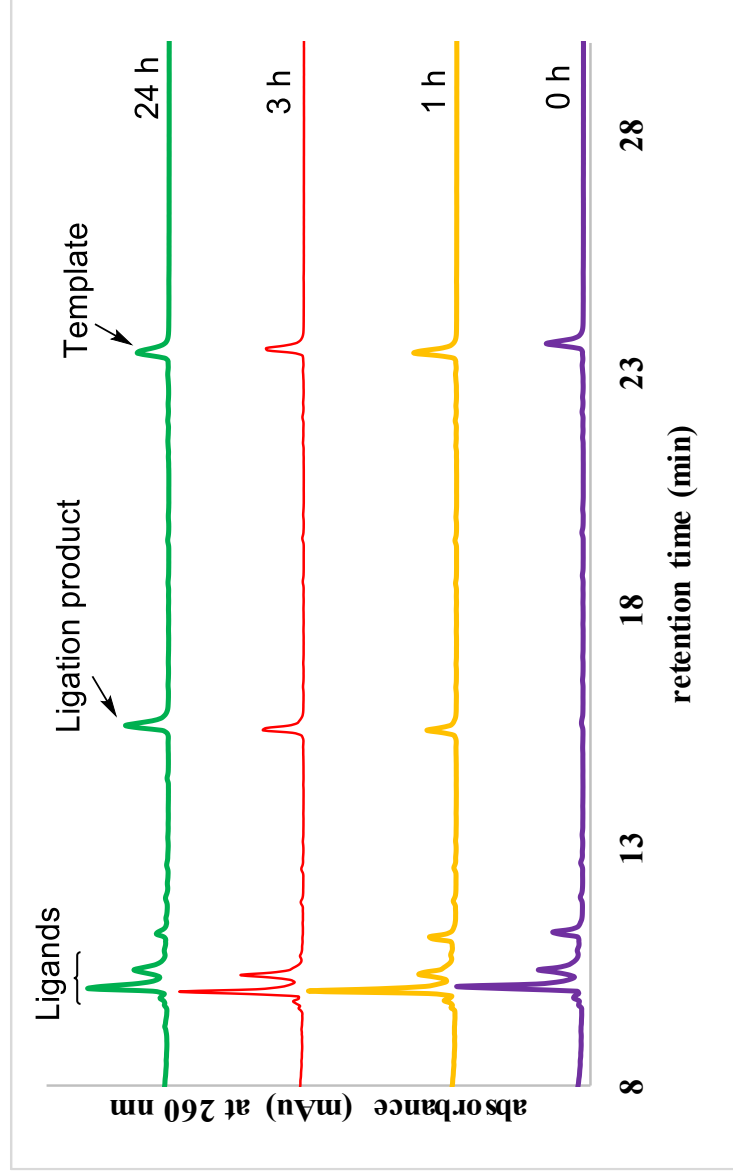
Ligation reaction: RNA ligands (R_{L1}, R_{L2}) on RNA template (R_{T1})



Entry	Sequence	Expected mass	Observed mass
Ligand R_{L1}	5'-AAAAATTTA T^{NH_2} -3'	2847	2850
Ligand R_{L2}	5'-PO $_3^{2-}$ -ATTATTA-3'	2286	2287
Template R_{T1}	3'-UUUUUAAAUAUAAU-5'	5021	5023
Ligation product R_{P1}	5'-AAAAATTTA I (NH)ATTATTA-3'	5116	5118
Yield (%)	1 h 3 h 24 h		
	43% 81% 102%		

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.*

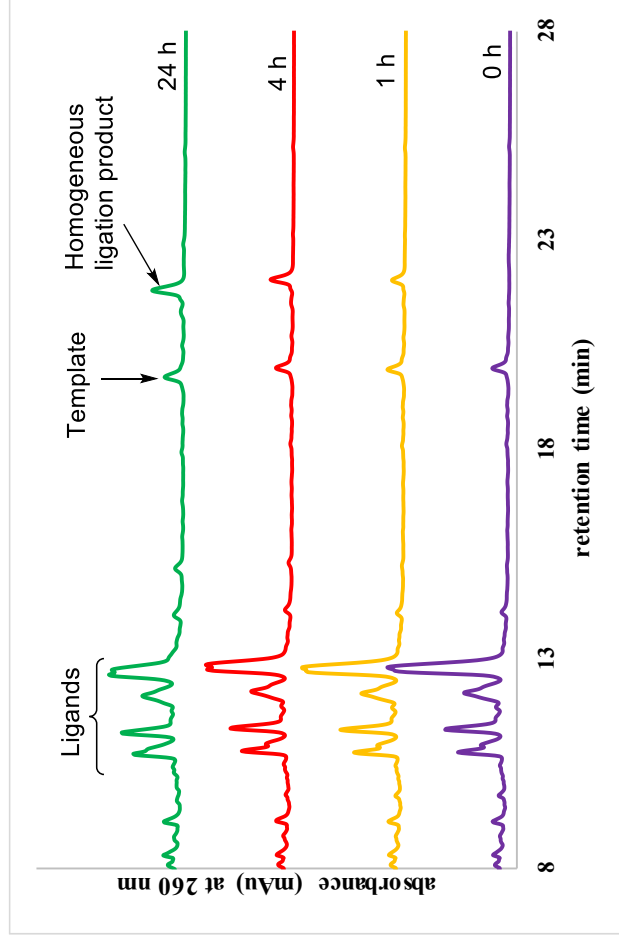
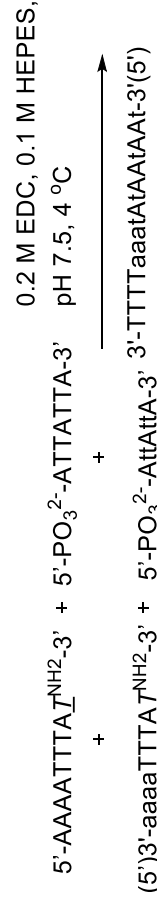
Ligation reaction: TNA ligands (T_{L1} , T_{L3}) on RNA template (R_{T1})



Entry	Sequence	Expected mass	Observed mass
Ligand T_{L1}	3'-aaaatttat- PO_3^{2-} -2'	2674	2678
Ligand T_{L3}	3'- NH_2 attatta-2'	1995	1998
Template R_{T1}	3'-UUUUAAAUAUAAUAAU-5'	5021	5026
Ligation product T_{P2}	3'-aaaatttat(NH)attatta-2'	4651	4656
Yield (%)	1 h 3 h 24 h		
	59% 100% 105%		

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.*

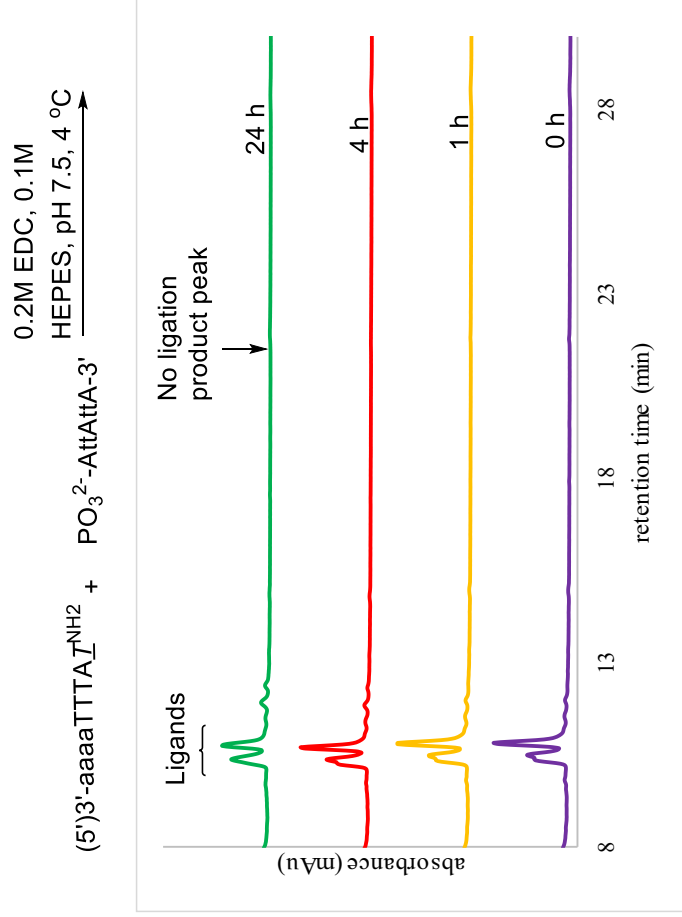
Ligation reaction: Competition among TRNA (C_{L1} , C_{L2}) and RNA ligands (R_{L1} , R_{L2}) on chimeric TRNA template (C_{T1})



Entry	Sequence	Expected mass	Observed mass
Ligand R_{L1}	5'-AAAAATTTA $\underline{T}^{\text{NH}_2}$ -3'	2847	2846
Ligand R_{L2}	5'- PO_3^{2-} -ATTATTA-3'	2286	2284
Ligand C_{L1}	(5')3'-aaaaTTTA $\underline{T}^{\text{NH}_2}$ -3'	2727	2726
Ligand C_{L2}	5'- PO_3^{2-} -AttAttA-3'	2166	2165
Template C_{T1}	3'-TTTTaaatAtAAatAAat-3'(5')	4923	4920
Homogeneous	5'-AAAAATTTA $\underline{T}^{\text{NH}_2}$ (NH)ATTATTA-3'	5116	5113
Ligation product R_{P1}	1 h 4 h 24 h		
	47% 86% 107%		
Heterogeneous	(5')3'-aaaaTTTA $\underline{T}^{\text{NH}_2}$ (NH)AttAttA-3'	4876	Not Observed
Ligation product C_{P1}			

Supplementary Figure 24. AEC traces of the competitive ligation reaction of RNA (R_{L1} , R_{L2}) and chimeric TNA-RNA (C_{L1} , C_{L2}) ligands (0.2 mM of each) on TNA-RNA chimeric template (C_{T1} , 0.1 mM) in 0.2M EDC, 0.1M 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.*

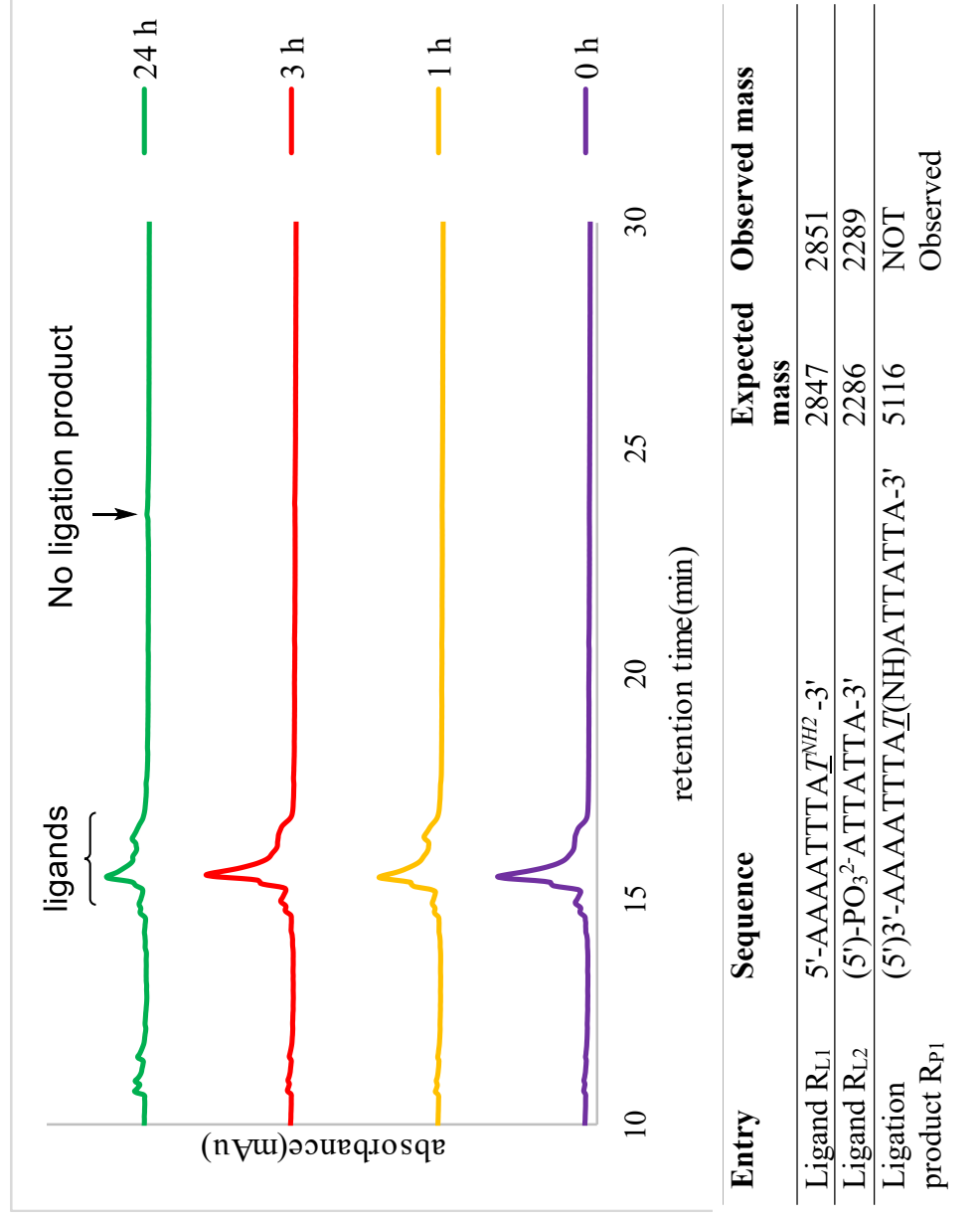
Control reaction: TRNA chimeric ligands (C_{L1} , C_{L2}) in absence of any template



Entry	Sequence	Expected mass	Observed mass
Ligand C_{L1}	$(5')3'-aaaaTTTA\overline{T}^{NH2}$	2727	2728
Ligand C_{L2}	$(5')\text{-}PO_3^{2-}\text{-AttAttA-}3'$	2166	2166
Ligation product C_{P1}	$(5')3'-aaaaTTTA\overline{T}^{(NH)}\text{AttAttA-}3'$	4876	NOT Observed

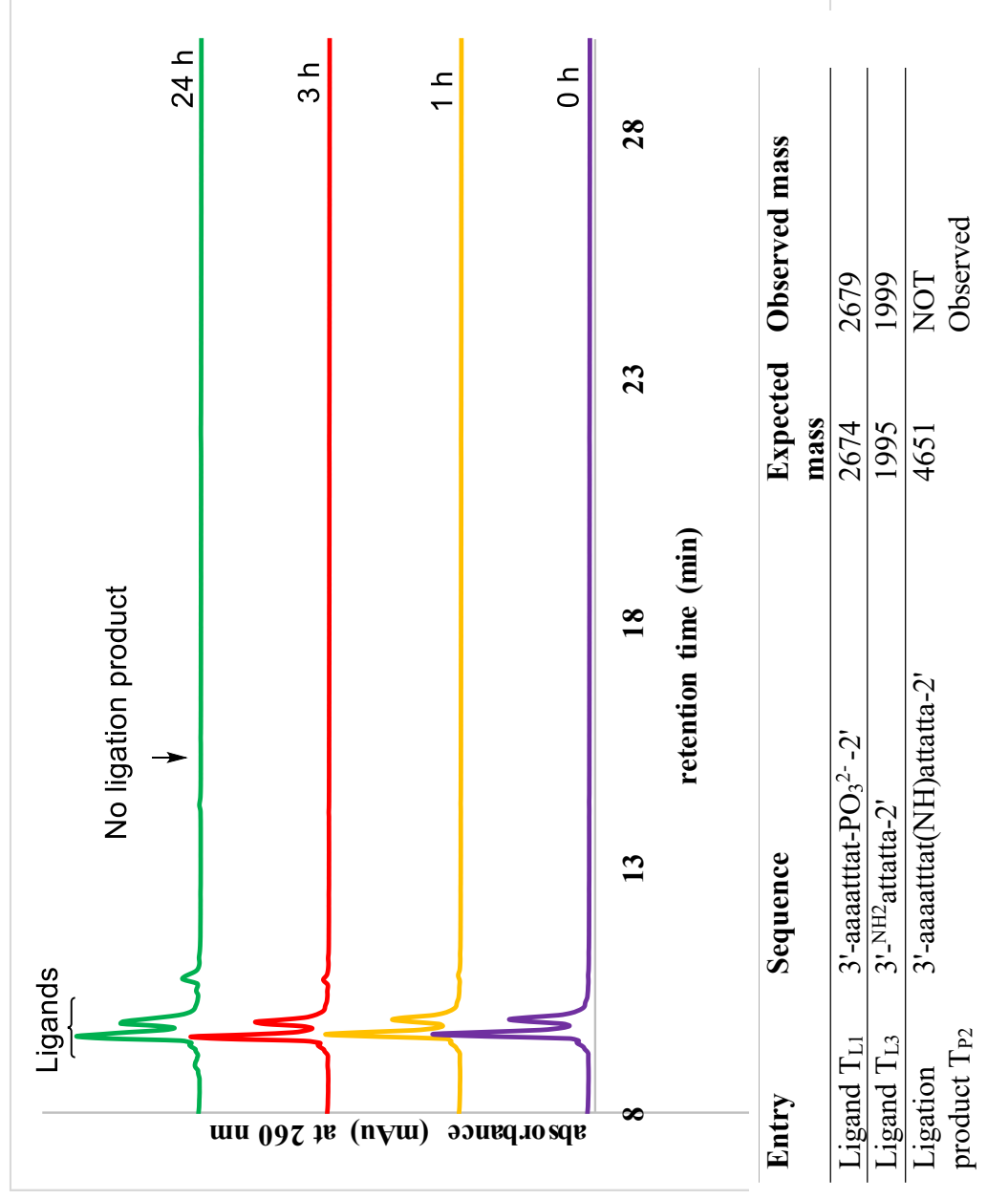
Supplementary Figure 25. AEC traces of the background ligation reaction of TNA-RNA chimeric ligands (0.2 mM of each) in absence of any template in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate that even after 24 h of reaction no peak corresponds to the ligation product is detected (Top 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



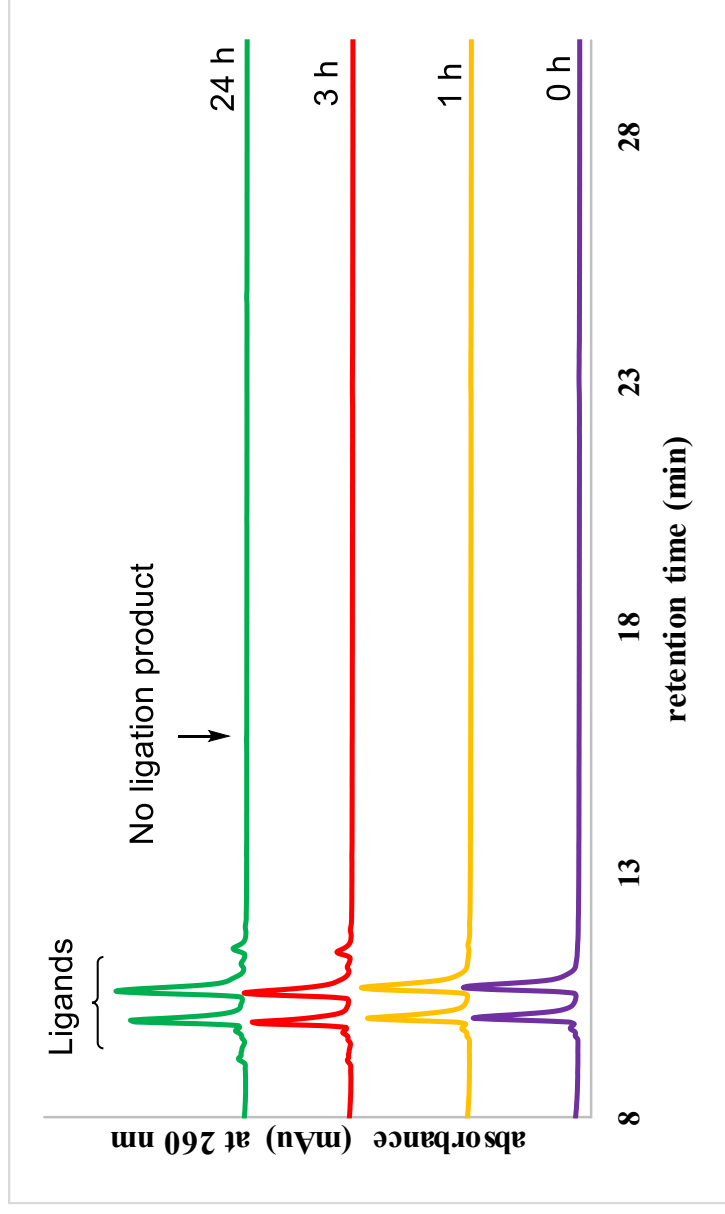
Supplementary Figure 26. AEC trace of the background ligation reaction of homogeneous RNA ligands (0.2 mM of each) in absence of any template in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate that even after 24 h of reaction no peak corresponds to the ligation product is detected (Top trace). 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



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



Entry	Sequence	Expected mass	Observed mass
Ligand T_{L1}	3'-aaaatttat- PO_3^{2-} -2'	2674	2676
Ligand T_{L2}	3'-OH ^{attatta} -2'	1994	1997
Ligation product T_{PI}	3'-aaaatttatattatta-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'-UUUU <u>AAAT</u> ^{NH2} -3'	10.1 ^a	2405	2406
2	5'-UUUUAAA <u>T</u> ^{NH2} -3'	10.1 ^a	2453	2454
3	5'- <u>TTTTAAAT</u> ^{NH2} -3'	9.5 ^a	2397	2396
4	5'-AUUAUUA <u>T</u> ^{NH2} -3'	10.2 ^a	2453	2453
5	5'-GUC <u>T</u> ^{NH2} -3'	14.5 ^b	1197	1198
6	5'-G <u>TC</u> ^{NH2} -3'	14.3 ^b	1195	1197
7	5'-UCG <u>T</u> ^{NH2} -3'	14.4 ^b	1197	1199
8	5'-PO ₃ ²⁻ -ACGA-3'	14.4 ^b	1326	1327
9	5'-PO ₃ ²⁻ -A <u>C</u> G <u>A</u> -3'	14.4 ^b	1294	1295
10	5'-PO ₃ ²⁻ -AGAC-3'	14.3 ^b	1326	1327
11	5'-UUUU <u>AAAT</u> (NH) <u>AUAAUAAU</u> -3'	20.6 ^c	4890	4886
12	5'-UUUUAAA <u>T</u> (NH)AUAAUAAU-3'	22.2 ^c	5018	5015
13	5'-TTTTAAAT(NH)AUAAUAAU-3'	25.5 ^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 NaClO₄, pH 8.2 as Buffer B; ^b 0 to 100% buffer B gradient using 10 mM Na₂HPO₄, pH 11 as Buffer A and 10 mM Na₂HPO₄, 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 Na₂HPO₄, pH 11 as Buffer A and 10 mM Na₂HPO₄, 1 M NaCl, pH 11 as Buffer B; ; peak purity (260 nm) ≥ 95%; desalting on Sep-Pak cartridges.^{S2} *MALDI-TOF spectrum was recorded using THAP matrix; A,U, G,C = RNA; A,T,C = DNA

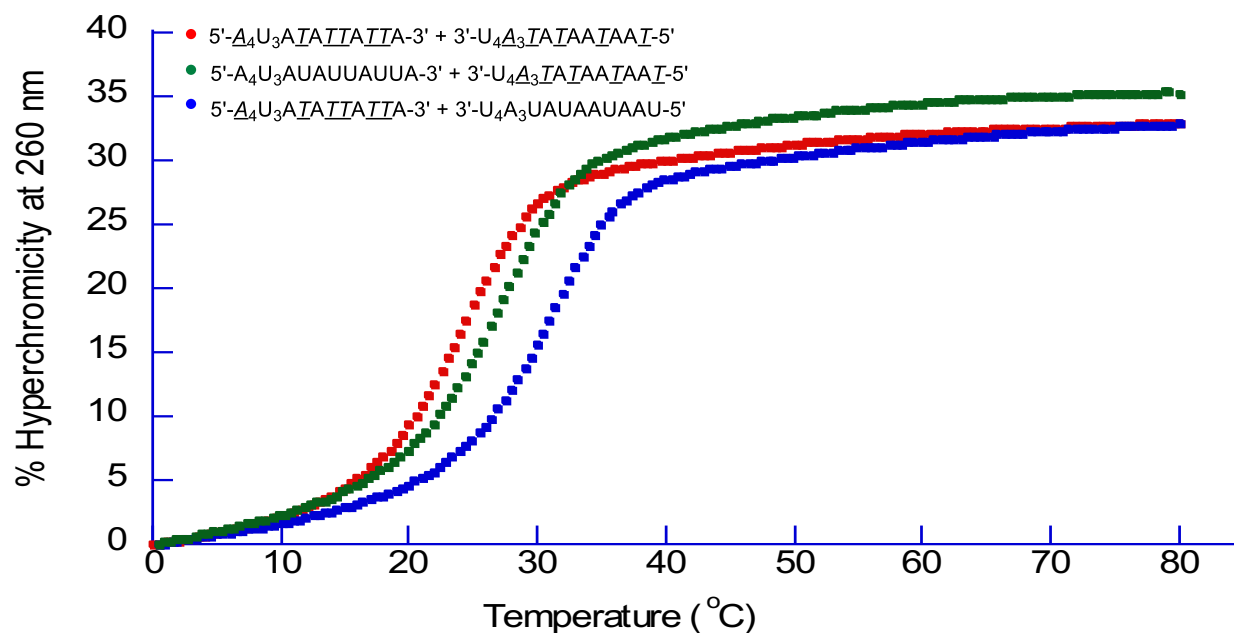
Supplementary Table 5: Thermal stability of duplexes (c = 2+2 μ M) formed from RDNA chimeric sequences and the corresponding parent DNA and RNA sequences.

Entry	Duplex	Description	UV-T _m (°C) ^b	ΔT_m (°C) ^{#,b}
AU, <u>AT</u> and parent RNA sequences				
1.	5'- <u>AAAA</u> UUU <u>ATATT</u> TA-3'+ 3'-UUUU <u>AAATATA</u> TAAT-5'	RDNA RDNA	32.8 ^a 23.6	-12.6
2.	5'- <u>AAAA</u> UUU <u>ATTTA</u> <u>TT</u> A-3'+ 3'-UUUUAAAUAUAAUAAU-5'	RDNA RNA	39.2 ^a 30.7	-5.7
3.	5'-AAAAUUUAUAUUAAUUA-3'+ 3'-UUUU <u>AAA</u> <u>TAT</u> AA <u>T</u> AA <u>T</u> -5'	RNA RDNA	35.2 ^a 26.5	-9.9
4.	5'-UUUU <u>AAAU</u> <u>AUAAU</u> <u>AAU</u> -3'+ 3'- <u>AAAA</u> UUU <u>AU</u> <u>AUU</u> <u>AUU</u> <u>A</u> -5'	RDNA RDNA	19.9 ^a 10.6 ^{b,c} <5 ^d	-24.8
5.	5'-UUUU <u>AAAT</u> (NH) <u>AUAAU</u> <u>AAU</u> -3'+ 3'- <u>AAAA</u> UUU <u>AU</u> <u>AUU</u> <u>AUU</u> <u>A</u> -5'	RDNA ^(NH) RDNA	11.6	-23.8
6.	5'-UUUUAAAUAUAAUAAU-3'+ 3'- <u>AAAA</u> UUU <u>AU</u> <u>AUU</u> <u>AUU</u> <u>A</u> -5'	RNA RDNA	36.3 ^a 27.2 ^{b,c} 18.6 ^d	-8.2
7.	5'-UUUUAAAT(NH)AUAAUAAU-3'+ 3'- <u>AAAA</u> UUU <u>AU</u> <u>AUU</u> <u>AUU</u> <u>A</u> -5'	RNA ^(NH) RDNA	27.9	-7.5
8.	5'-UUUU <u>AAAU</u> <u>AUAAU</u> <u>AAU</u> -3'+ 3'-AAAAUUUAUAUUAUUA-5'	RDNA RNA	20.1 ^{b,c}	-15.3
9.	5'-UUUU <u>AAAT</u> (NH) <u>AUAAU</u> <u>AAU</u> -3'+ 3'-AAAAUUUAUAUUAUUA-5'	RDNA ^(NH) RNA	21.5	-13.9
10.	5'- <u>TTT</u> <u>TA</u> <u>AAT</u> <u>AT</u> <u>ATAAT</u> -3'+ 3'- <u>AAAA</u> UUU <u>AU</u> <u>AUU</u> <u>AUU</u> <u>A</u> -5'	DNA RDNA	15.8 ^{b,c}	-19.6
11.	5'-UUUU <u>AAAT</u> <u>AUAAU</u> <u>AAU</u> -3'+ 3'- <u>AAAA</u> UUU <u>AU</u> <u>AUU</u> <u>AUU</u> <u>A</u> -5'	RDNA RDNA	19 ^a <5 ^d	
12.	5'-UUUUAAAT <u>TA</u> UAAUAAU-3'+ 3'- <u>AAAA</u> UUU <u>AU</u> <u>AUU</u> <u>AUU</u> <u>A</u> -5'	RNA RDNA	35 ^a 14.9 ^d	

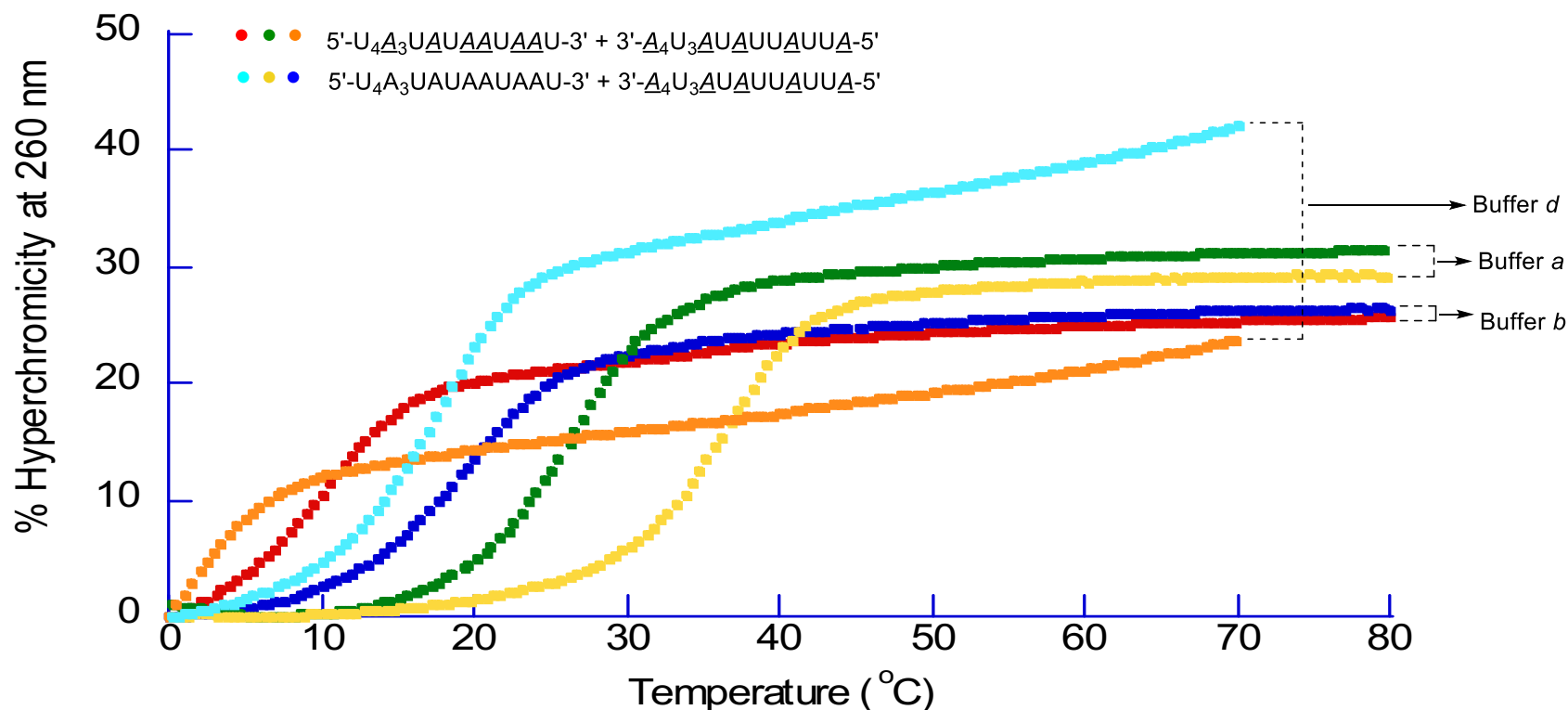
Entry	Duplex	Description	UV-T _m (°C) ^b	ΔT _m (°C) ^{#,b}
AUGC, <u>ATGC</u> sequences and parent RNA sequences				
13.	5'-UUUU <u>AAGUACAGUAAU</u> -3'+ 3'- <u>AAAAUUCAGUCUU</u> <u>A</u> -5'	RDNA RDNA	26.7	-24.6
14.	5'-UUUU <u>AAGUACAGUAA</u> U-3'+ 3'-AAAAUUCAUGUCAUUA-5'	RDNA RNA	36.9	-14.4
15.	5'-UUUUAAGUACAGUAAU-3'+ 3'- <u>AAAAUUCAGUCUU</u> <u>A</u> -5'	RNA RDNA	44.1	-7.2
16.	5'-G <u>TCTACG</u> <u>A</u> -3' + 3'- <u>CAGAT</u> <u>GCU</u> -5'	RDNA RDNA	18.6	-21.5
17.	5'-G <u>TCTACG</u> <u>A</u> -3' + 3'-CAGAUGCU-5'	RDNA RNA	31.8	-8.3
18.	5'-GUCUACGA-3' + 3'- <u>CAGATGCU</u> -5'	RNA RDNA	27.6	-12.5
RNA series for comparison				
19.	5'-AAAAUUUAUUAUUA-3'+ 3'-UUUUAAAUAUAAUAAU-5'	RNA RNA	36.4 ^{b,c}	
20.	5'-UUUUAAAUAUAAUAAU-3'+ 3'-AAAAUUUAUUAUUA-5'	RNA RNA	35.4 ^{b,c}	
21.	5'-UUUUAAGUACAGUAAU-3'+ 3'-AAAAUUCAUGUCAUUA-5'	RNA RNA	51.3	
22.	5'-GUCUACGA-3' + 3'-CAGAUGCU-5'	RNA 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.

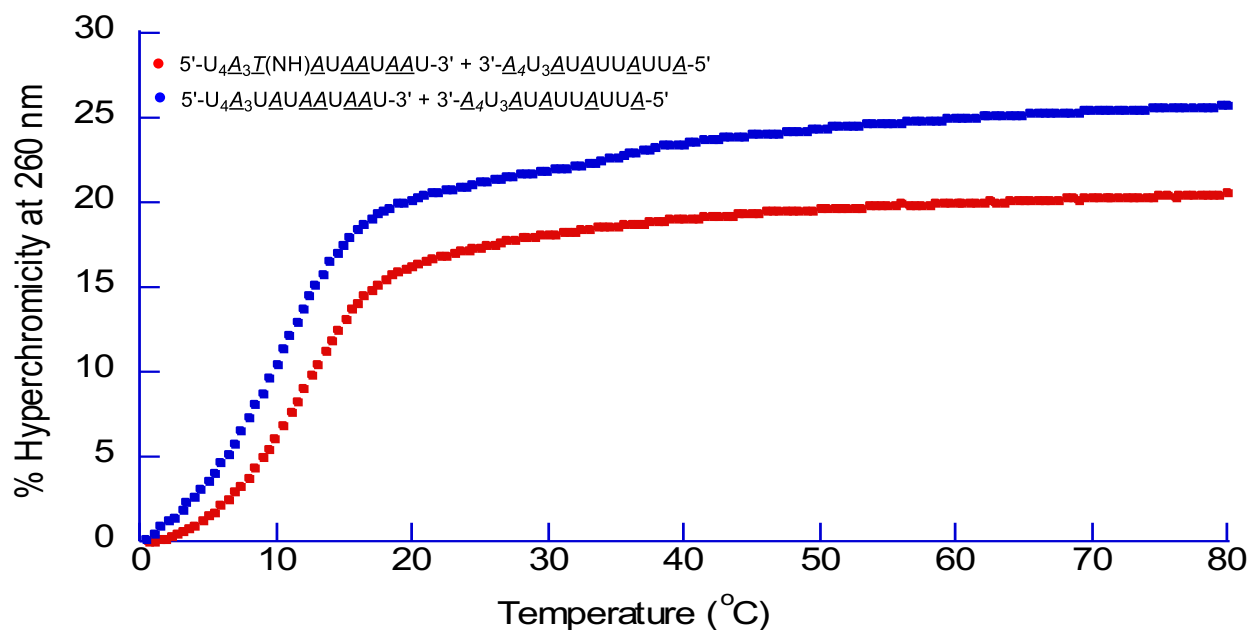
UV melting curves



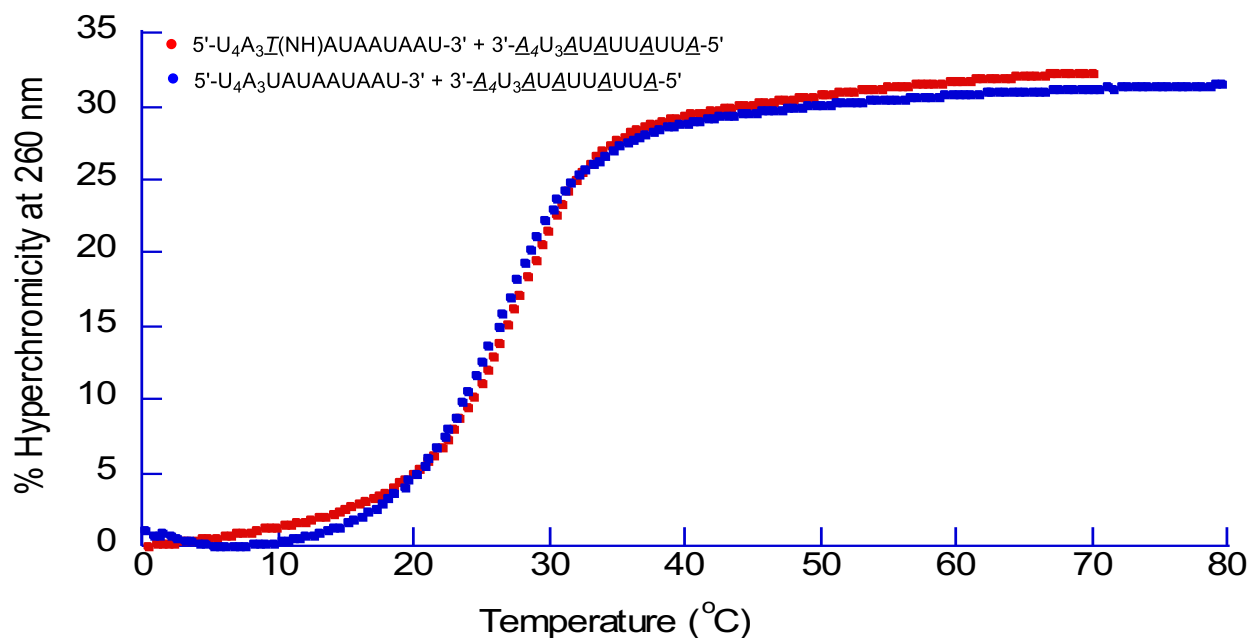
Supplementary Figure 29. Comparison of UV- T_m curves of duplexes formed by non-self-complementary RDNA chimeric strand with its complementary RNA sequence versus the duplex formed with its complementary RDNA chimeric strand (Supplementary Table 5, Entry 1-3) in 10 mM Na₂HPO₄, and 150 mM NaCl, 100 μ M EDTA at pH 7.2 buffer. A, U = RNA; A, T = DNA.



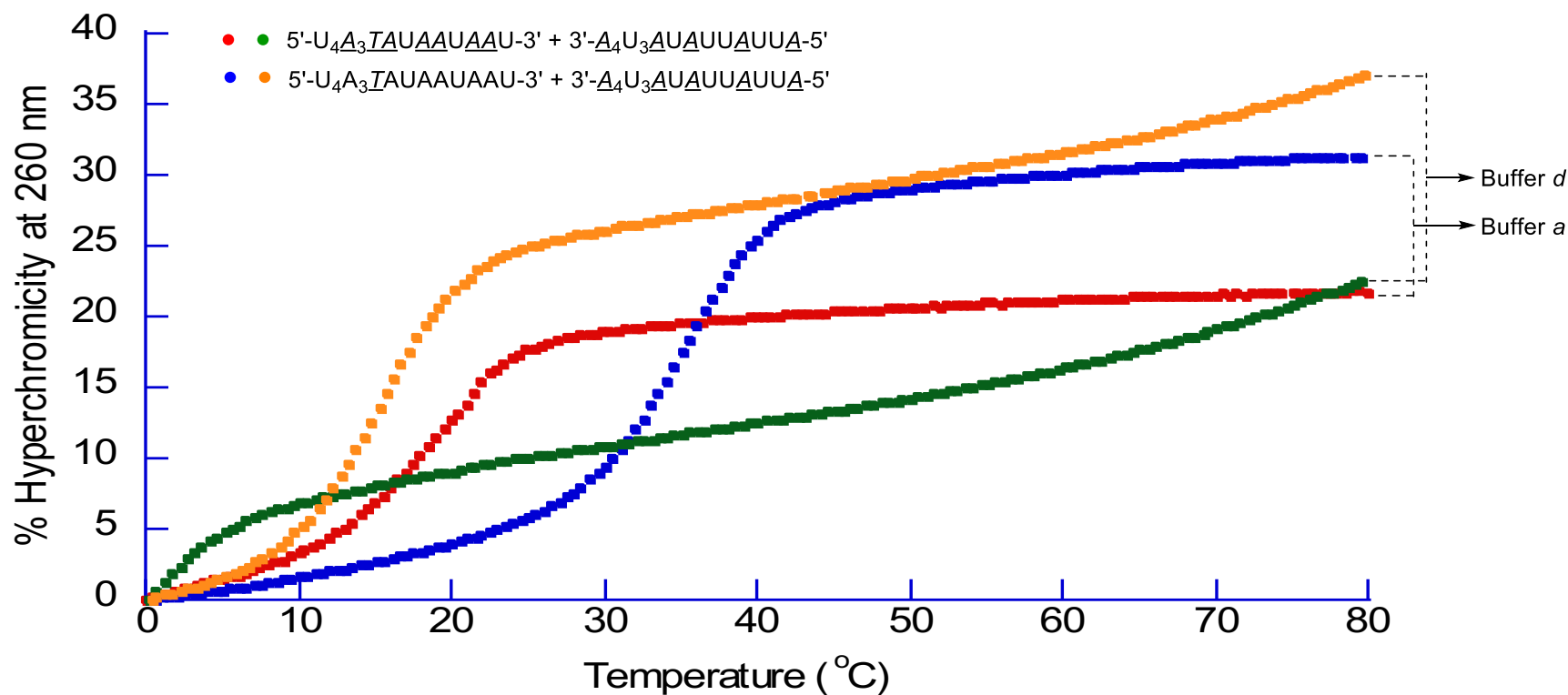
Supplementary Figure 30. Effect of salt concentration on UV- T_m curves of duplexes formed by non-self-complementary RDNA chimeric strand in 10 mM Na₂HPO₄, 100 μ M EDTA, pH 7.2 (150 mM and 1M NaCl, Supplementary Table 5, Entry 4, 6 buffers *a*, *b* respectively) and in 0.1 M HEPES at pH 7.5 (no salt, Supplementary Table 5, Entry 4,6, buffer *d*). A, U = RNA; A = DNA.



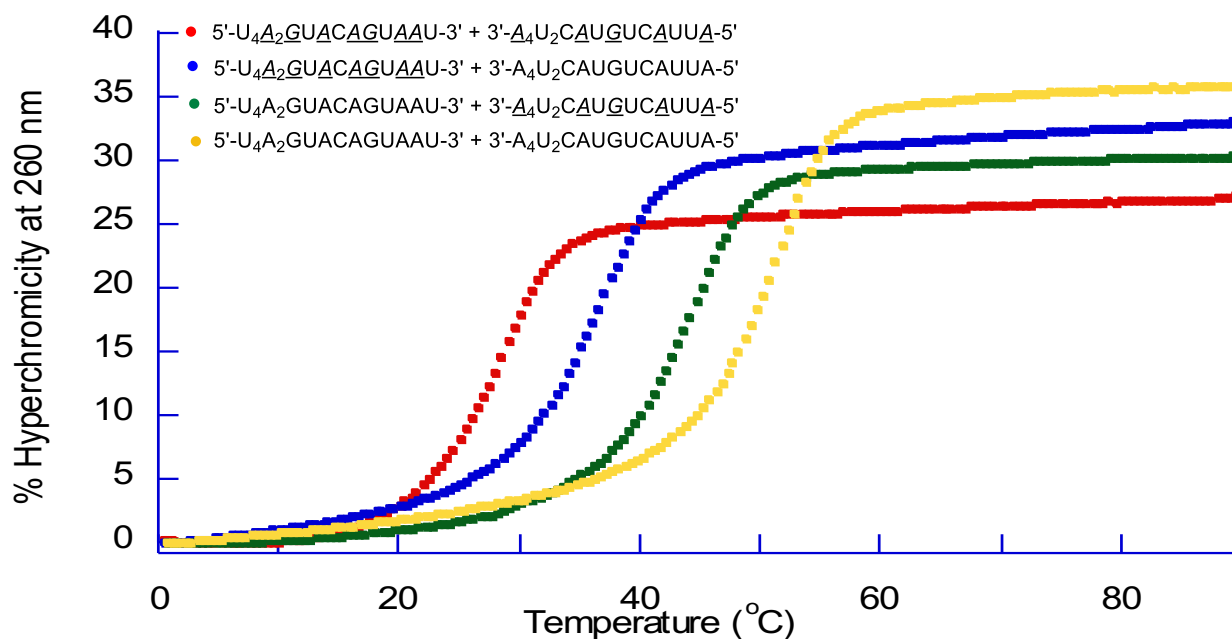
Supplementary Figure 31. Comparison of UV-T_m curves of duplexes formed from RDNA chimeric sequences with phosphodiester linkage (blue curve) and with corresponding *I* phosphoramidate linkage (red curve), demonstrating similar thermal stability. (Supplementary Table 5, Entry 4-5) in 10 mM Na₂HPO₄, and 150 mM NaCl, 100 μM EDTA at pH 7.2 buffer.



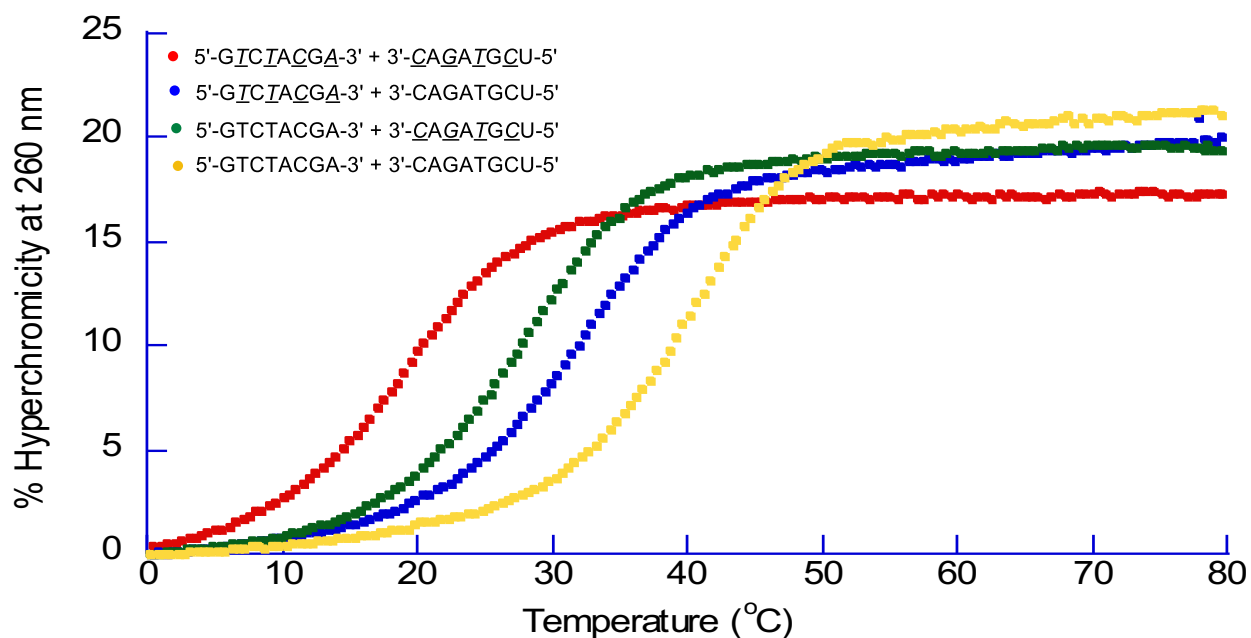
Supplementary Figure 32. Comparison of UV-T_m curves of duplexes formed from RDNA chimeric sequences and its complementary homogeneous RNA strand, with phosphodiester linkage (blue) and with corresponding I phosphoramidate linkage (red) demonstrating similar thermal stability. (Supplementary Table 5, Entry 6-7) in 10 mM Na₂HPO₄, and 150 mM NaCl, 100 μM EDTA at pH 7.2 buffer.



Supplementary Figure 33. Effect of salt concentration on UV- T_m 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 11-12, 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.

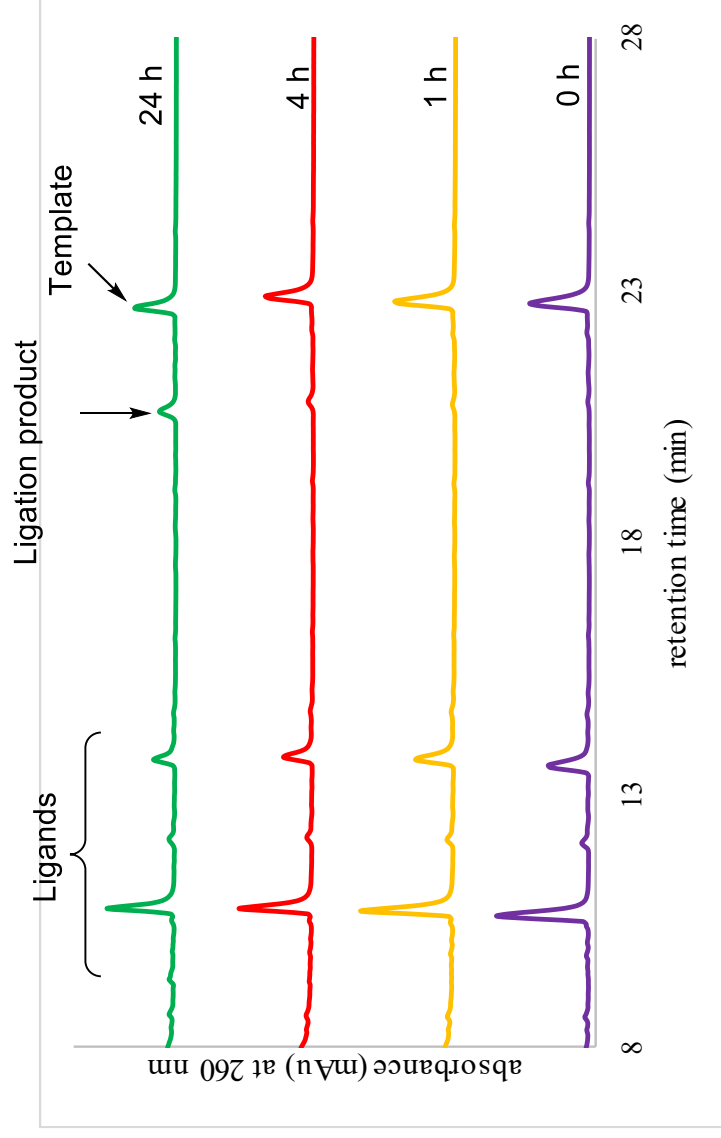
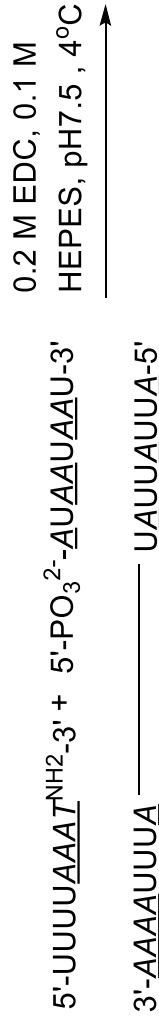


Supplementary Figure 34. Comparison of UV- T_m curves of non-self-complementary RDNA chimeric strands with its complementary homogeneous RNA and chimeric strands containing all four nucleobases (Supplementary Table 5, Entry 13-15,21) in 10 mM Na₂HPO₄, and 150 mM NaCl, 100 μ M EDTA at pH 7.2 buffer. A, U, G, C = RNA; A, G = 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_2HPO_4 , and 150 mM NaCl, 100 μM EDTA at pH 7.2 buffer. A, U, G, C = RNA; A, T, G, C = DNA.

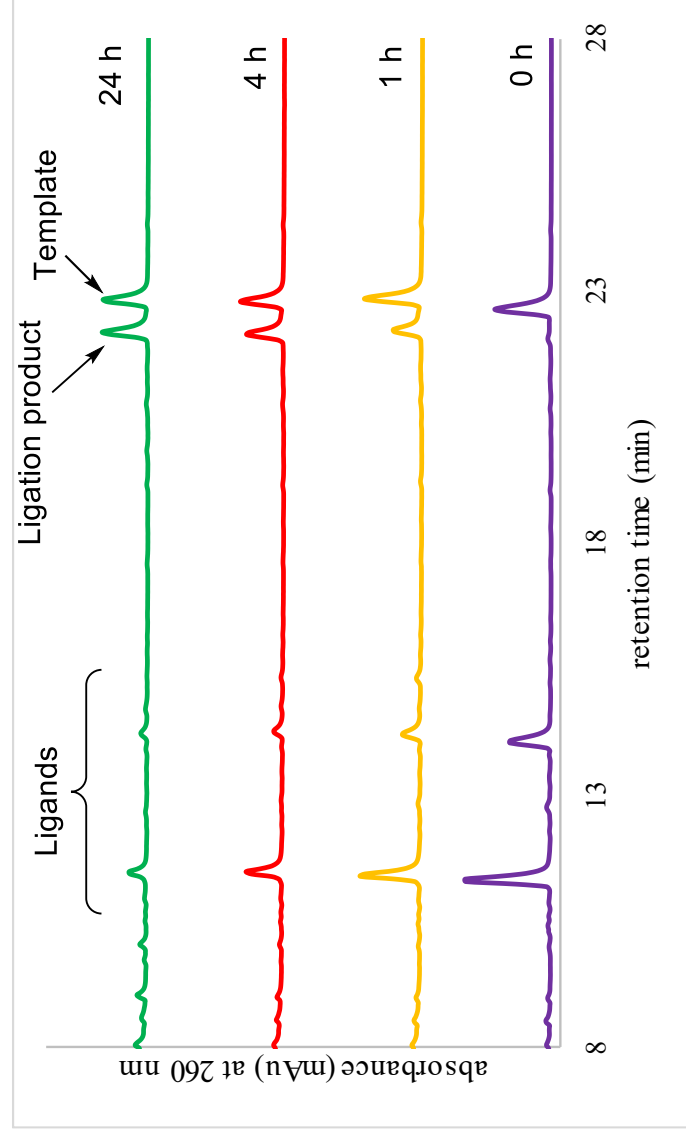
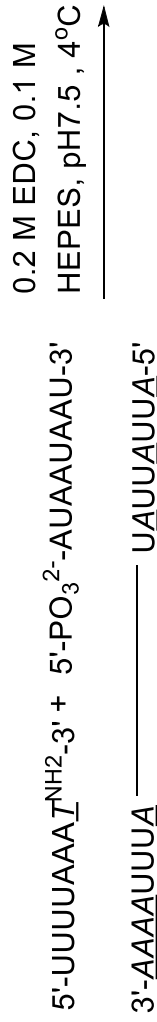
Ligation reaction: RDNA chimeric ligands on a RDNA chimeric template



Entry	Sequence	Expected mass	Observed mass
Ligand C _{L3}	5'-UUUUAAAAT ^{NH2}	2405	2407
Ligand C _{L4}	5'-PO ₃ ²⁻ -AUAAUAUU-3'	2502	2504
Template C _{T2}	3'-AAAAUUUAUUAAUU-5'	4893	4894
Ligation product C _{P2}	5'-UUUUAAAAT(NH)AUAAUAUU-3'	4890	coincidental with template peak
Yield (%)	1 h 4 h 24 h		
	<5% <10% 36%		

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.*

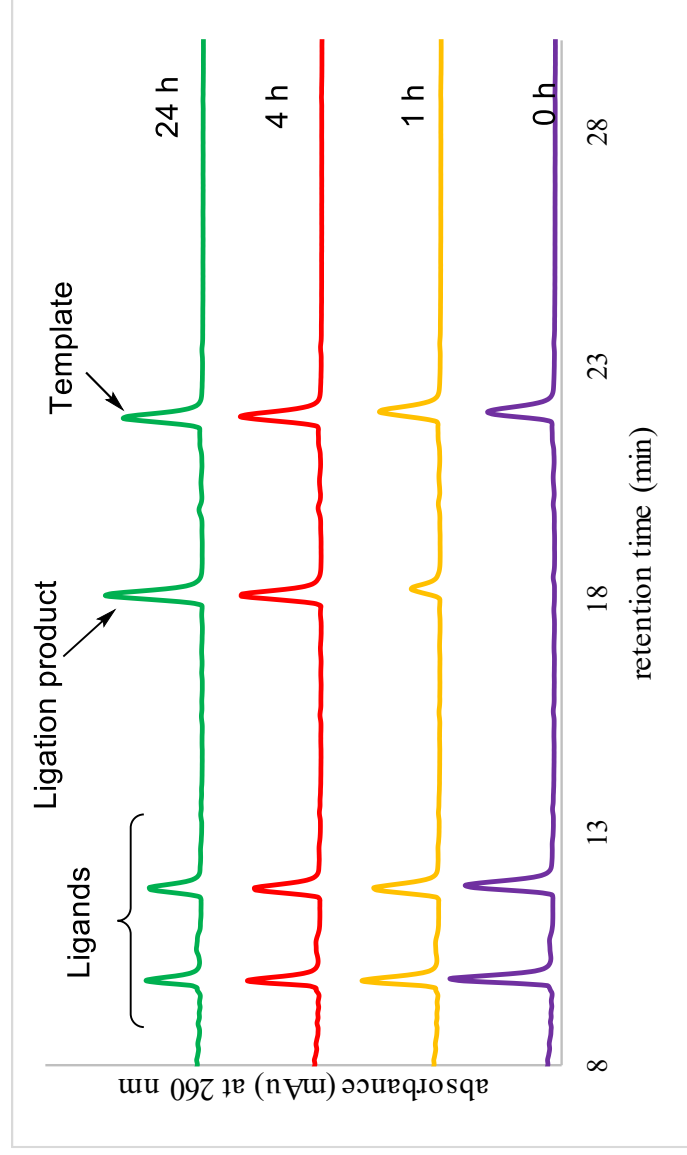
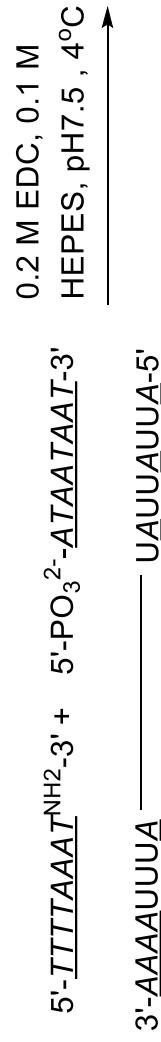
Ligation reaction: RNA ligands on a RDNA chimeric template



Entry	Sequence	Expected mass	Observed mass
Ligand R _{L3}	5'-UUUUAAA <u>T</u> ^{NH₂}	2453	2454
Ligand R _{L4}	5'-PO ₃ ²⁻ -AUAAUAAU-3'	2582	2583
Template C _{T2}	3'- AAAA UUUU <u>AU</u> UU <u>AU</u> U-5'	4893	4893
Ligation product R _{P2}	5'-UUUUAAA <u>T</u> (NH)AUAAUAAU-3'	5018	5018
Yield (%)	1 h	57%	91%
	24 h		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.*

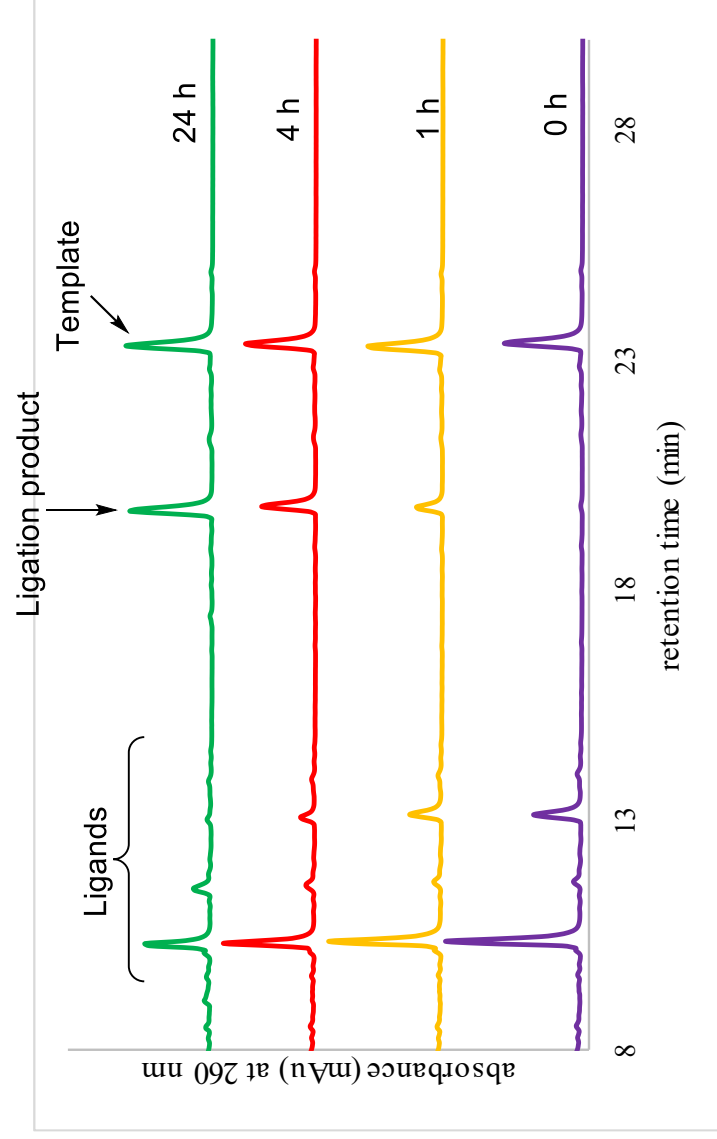
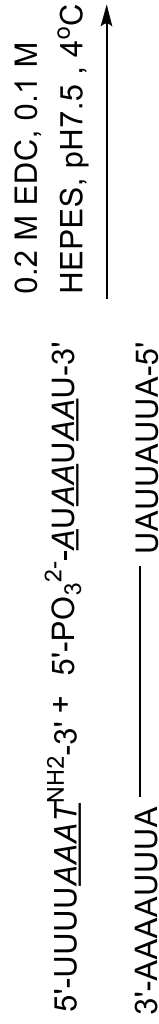
Ligation reaction: DNA ligands on a RDNA chimeric template



Entry	Sequence	Expected mass	Observed mass
Ligand D _{L1}	5'- <u>TTTTAAAT</u> ^{NH₂}	2397	2398
Ligand D _{L2}	5'-PO ₃ ²⁻ - <u>ATAATAAT</u> -3'	2496	2497
Template C _{T2}	3'- <u>AAAAUUUAUUUUUA</u> -5'	4893	4892
Ligation product D _{P1}	5'- <u>TTTTAAAT</u> (NH) <u>ATAATAAT</u> -3'	4876	4875
Yield (%)	1 h	4h	24 h
	51%	94%	107%

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.*

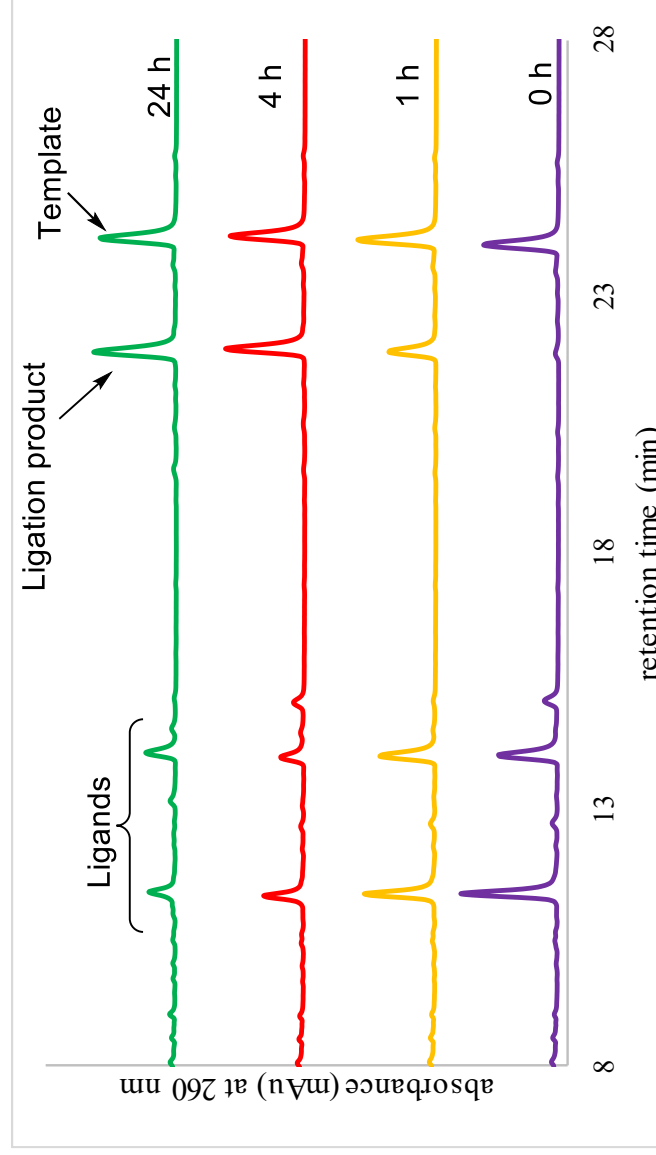
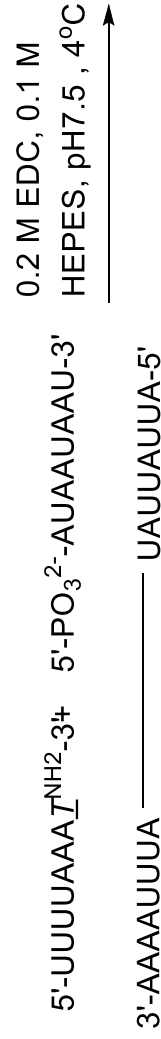
Ligation reaction: RDNA chimeric ligands on an RNA template



Entry	Sequence	Expected mass	Observed mass
Ligand CL ₃	5'-UUUUAAAT ^{NH₂}	2405	2405
Ligand CL ₄	5'-PO ₃ '-AUAAUAAU-3'	2502	2502
Template RT ₂	3'-AAAAUUUAUUUAUA-5'	5021	5019
Ligation product CP ₂	5'-UUUUAAAT(NH) <u>AUAAUAAU</u> -3'	4890	4888
Yield (%)	1 h 4 h 24 h		
	31% 73% 94%		

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.*

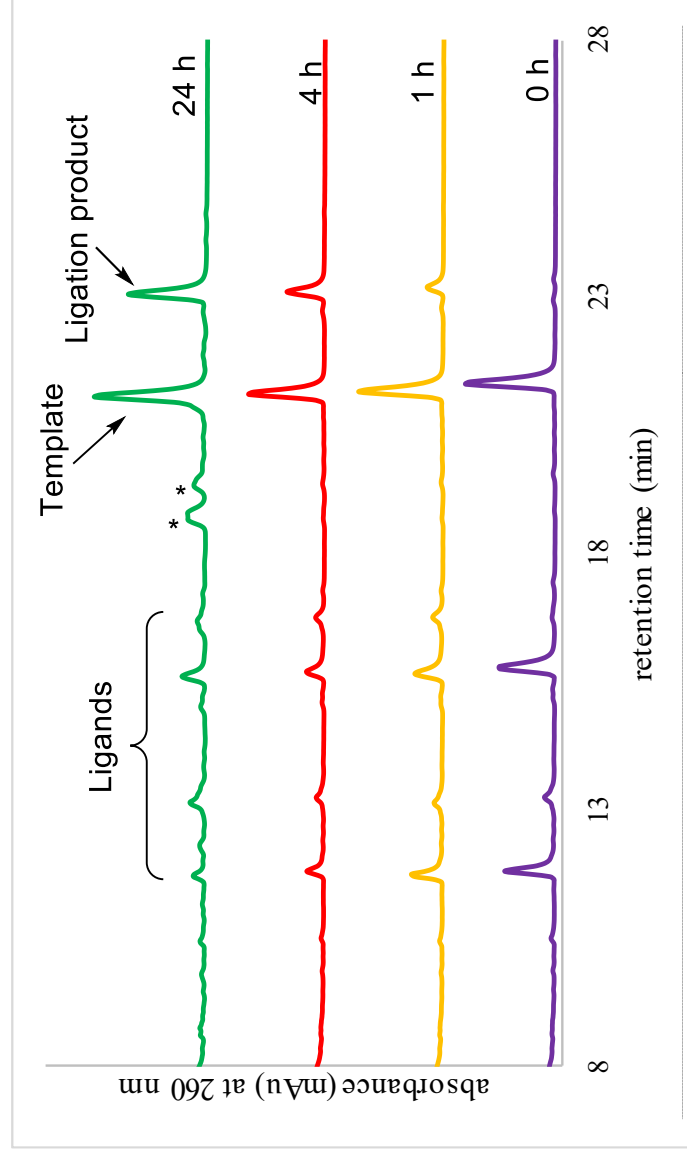
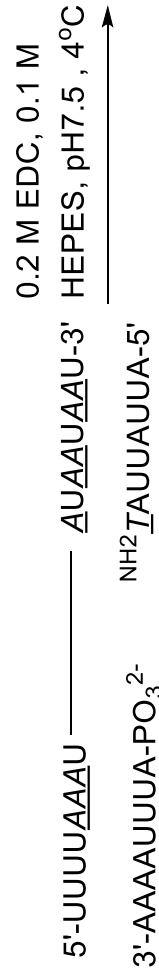
Ligation reaction: RNA ligands on an RNA template



Entry	Sequence	Expected mass	Observed mass
Ligand RL3	5'-UUUUAAA <u>T</u> ^{NH2}	2453	2453
Ligand RL4	5'-PO ₃ ²⁻ -AUAUAAU-3'	2582	2582
Template RT2	3'-AAAAUUUAUUAUUA-5'	5021	Assumed to be coincidental with template peak
Ligation product RP2	5'-UUUUAAA <u>T</u> (NH)AUAUAAU-3'	5018	5017
Yield (%)	1 h	4 h	24 h
	58%	106%	108%

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.*

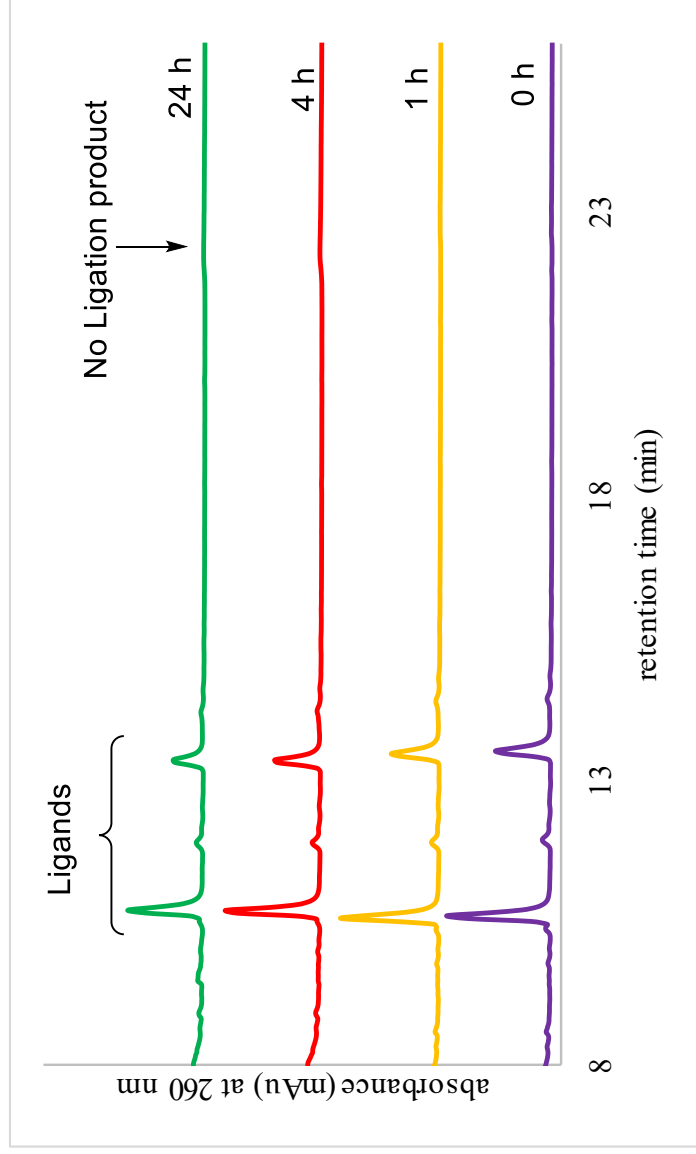
Ligation reaction: RNA ligands on a RDNA chimeric template



Entry	Sequence	Expected mass	Observed mass
Ligand R _{L5}	3'- ^{NH₂} TAUUUAUA-5'	2453	2453
Ligand R _{L6}	3'-AAAAUUUA-PO ₃ ²⁻ -5'	2582	Not detected
Template C _{T3}	5'-UUUUAAA <u>TAUAAU</u> AAU-3'	4891	4888
Ligation product R _{P3}	3'-AAAAUUUA(NH)TAUUUAUA-5'	5018	5016
Yield (%)	1 h 4 h 24 h		
	19% 49% 61%		

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.

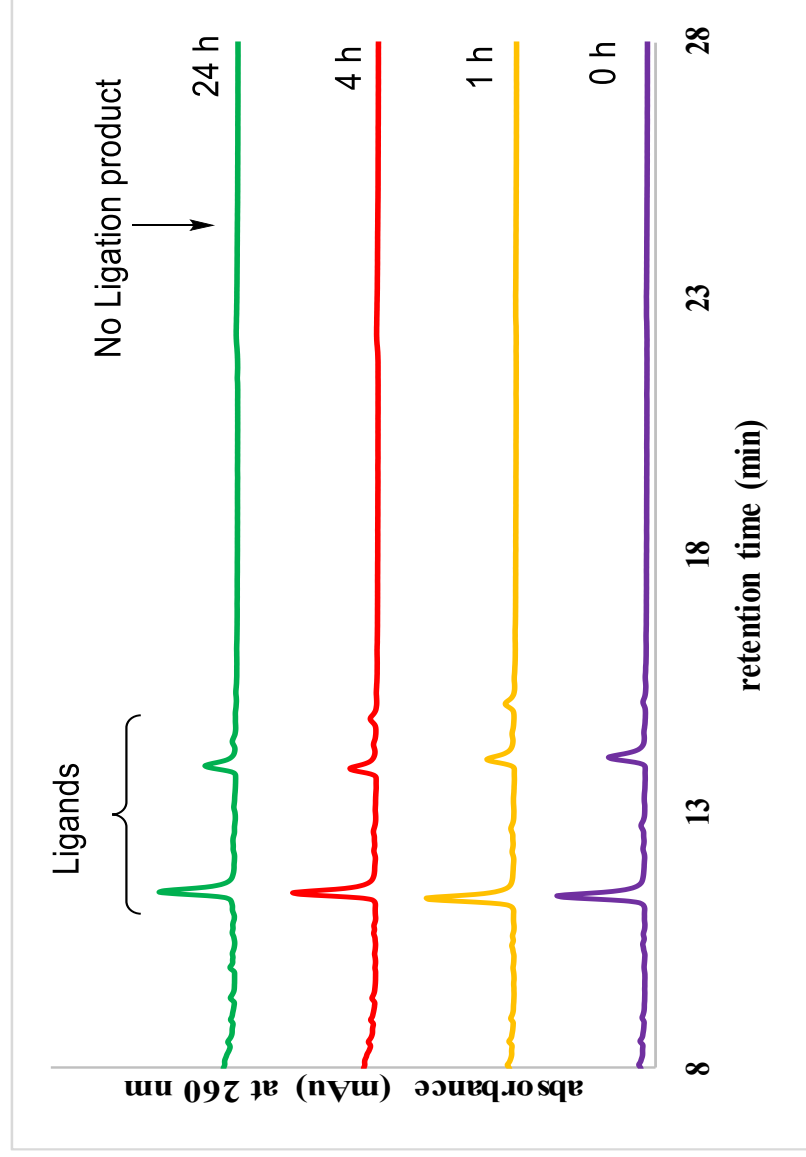
Control experiment: RDNA chimeric ligands in the absence of any template



Entry	Sequence	Expected mass	Observed mass
Ligand CL3	5'-UUUU <u>AAAT</u> ^{NH2}	2405	2405
Ligand CL4	5'-PO ₃ ²⁻ - <u>AUAAUAAU</u> -3'	2502	2502
Ligation product CP2	5'-UUUU <u>AAAT</u> (NH) <u>AUAAUAAU</u> -3'	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.*

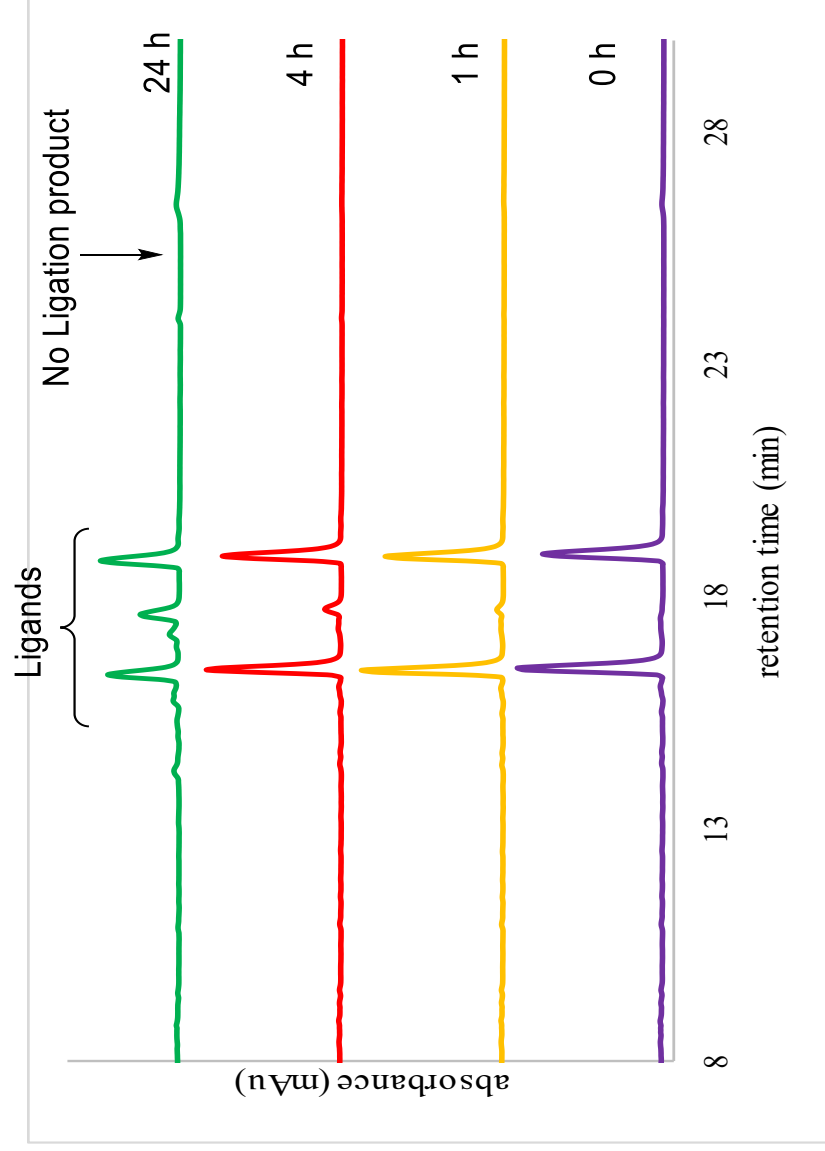
Control experiment: RNA ligands in the absence of any template



Entry	Sequence	Expected mass	Observed mass
Ligand R _{L3}	5'-UUUUAAA <u>T</u> ^{NH₂}	2453	2454
Ligand R _{L4}	5'-PO ₃ ²⁻ -AUAAUAAU-3'	2582	2583
Ligation product R _{P2}	5'-UUUUAAA <u>T</u> (NH)AUAAUAAU-3'	5018	Not Observed

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.*

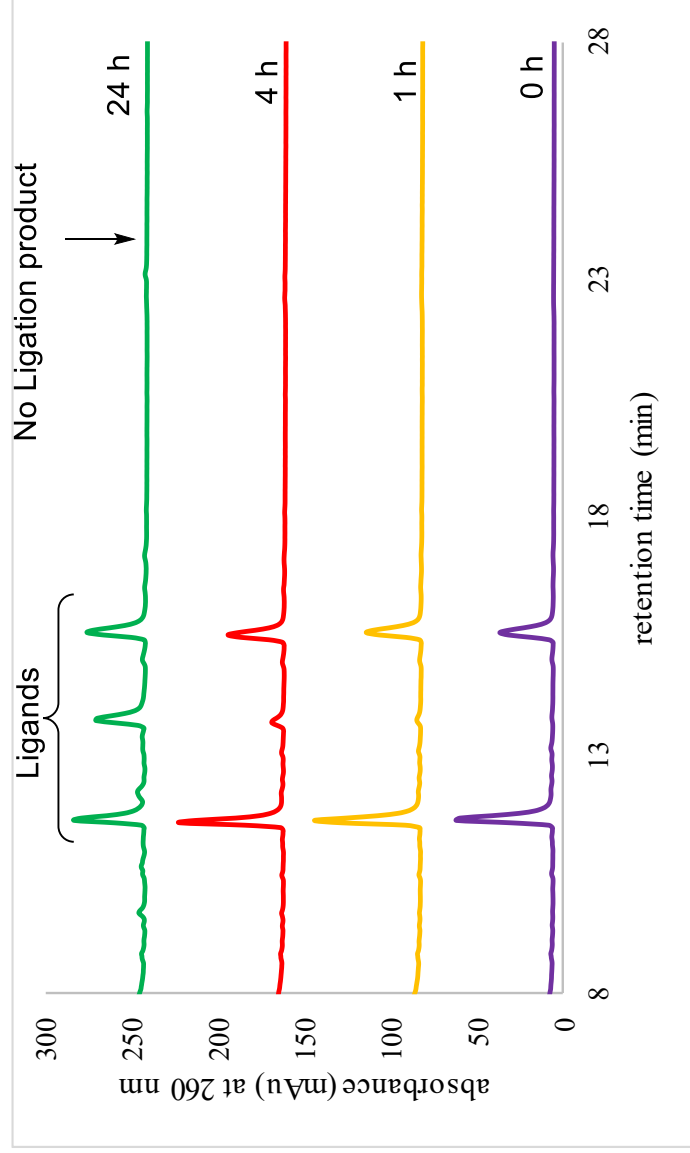
Control experiment: DNA ligands in the absence of template



Entry	Sequence	Expected mass	Observed mass
Ligand D _{L1}	5'- <u>TTTTAAAT</u> ^{NH₂}	2397	2396
Ligand D _{L2}	5'-PO ₃ ²⁻ - <u>ATAATAAT</u> -3'	2496	2495
Ligation product D _{P2}	5'- <u>TTTTAAAT</u> (NH) <u>ATAATAAT</u> -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



Entry	Sequence	Expected mass	Observed mass
Ligand R _{L5}	5'-AUUAUUA $\underline{T}^{\text{NH}_2}$	2453	2453
Ligand R _{L6}	5'-PO ₃ ²⁻ -AUUUAAAA-3'	2582	2582
Ligation product R _{P3}	5'-AUUAUUA $\underline{T}(\text{NH})$ AUUUAAAA-3'	5018	Not Observed

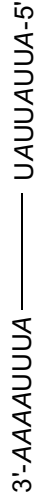
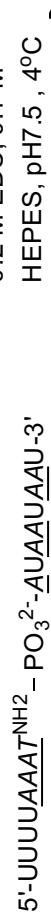
Supplementary Figure 45. AEC traces of the control of ligation reaction of RNA ligands (0.2 mM of each) in absence of any template with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. Even after 24 h of reaction no peak corresponding to the ligation product was detected (Top trace). MALDI of the 24 h reaction 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



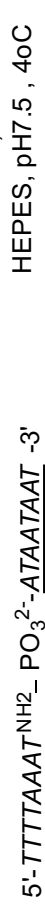
DNA-RNA chimeric template and RNA ligands

Vs

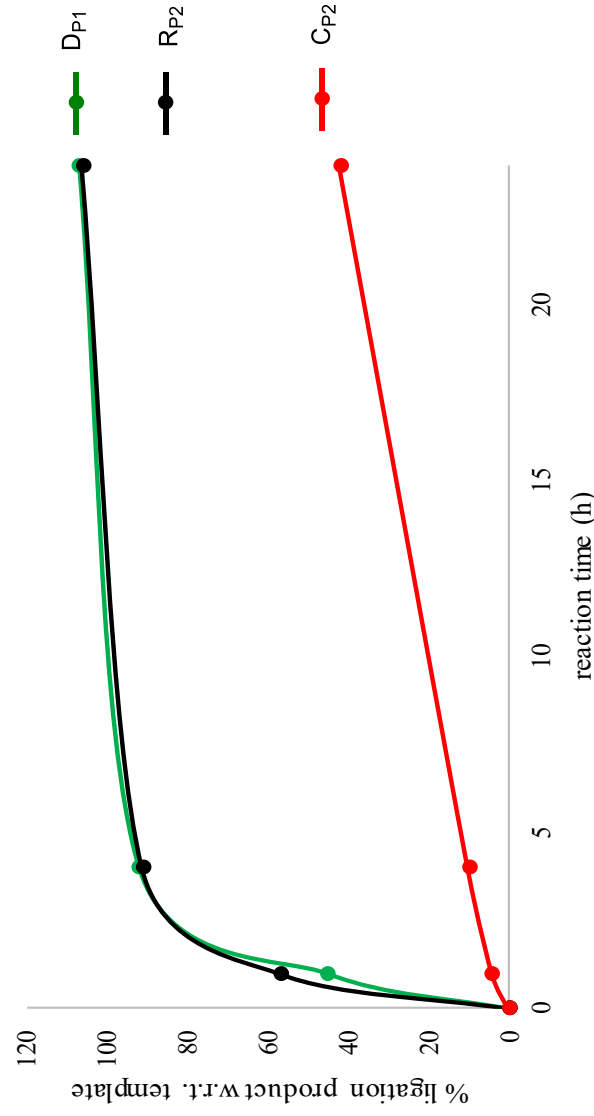


DNA-RNA chimeric template and chimeric ligands

Vs

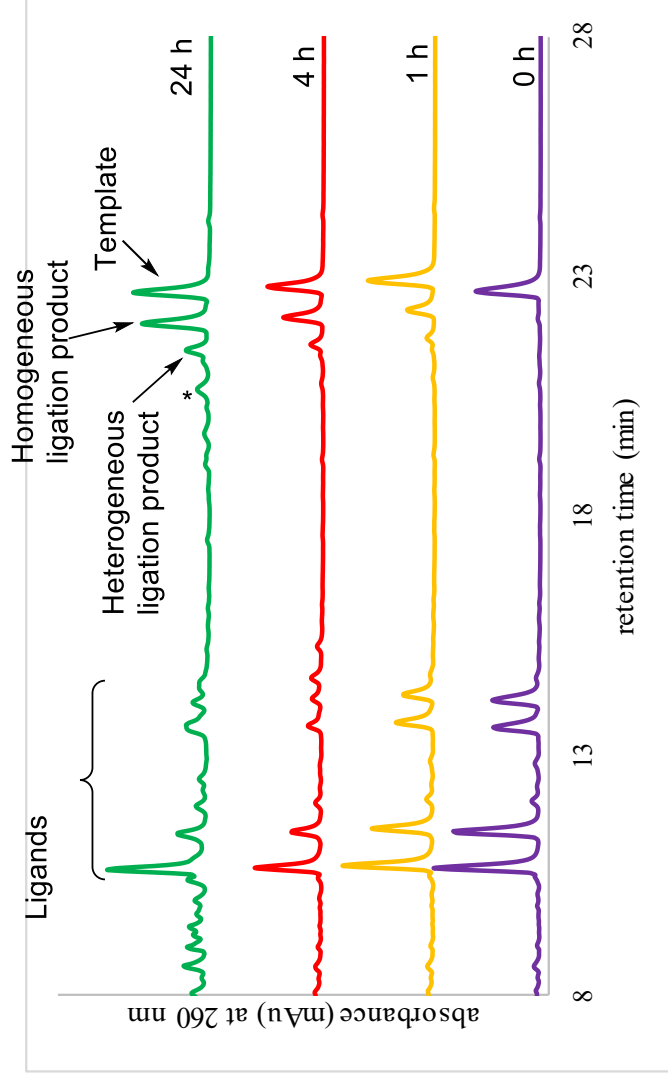


DNA-RNA chimeric template and DNA ligands



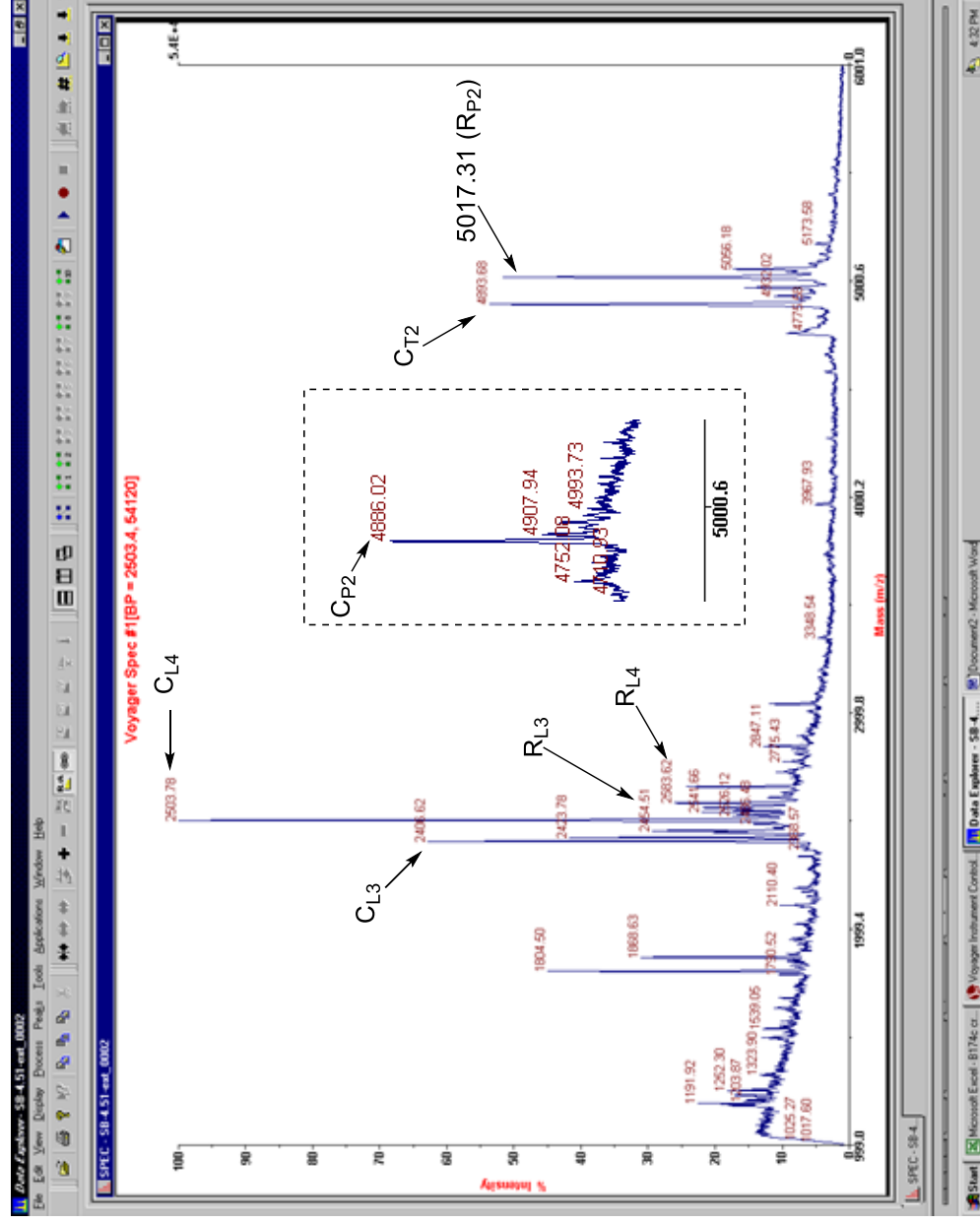
Supplementary Figure 46. Comparison of the rate of homogeneous and heterogeneous ligands (0.2 mM each) on RDNA chimeric template (0.1 mM). Rate of ligation with homogeneous ligands (DNA and RNA) are much faster (green and black line) than the corresponding heterogeneous ligands (red line). For conditions 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



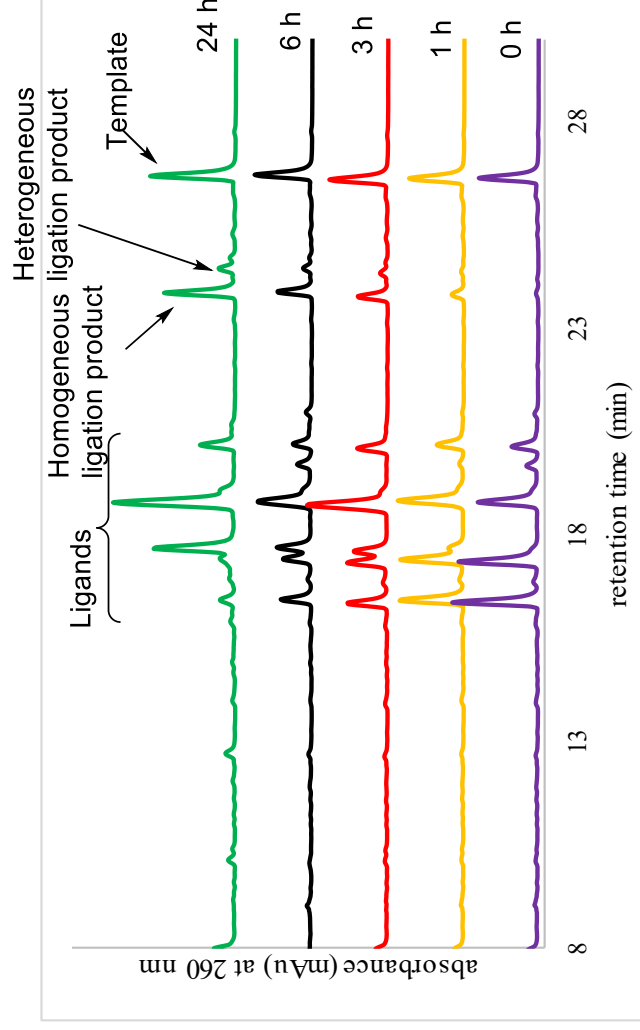
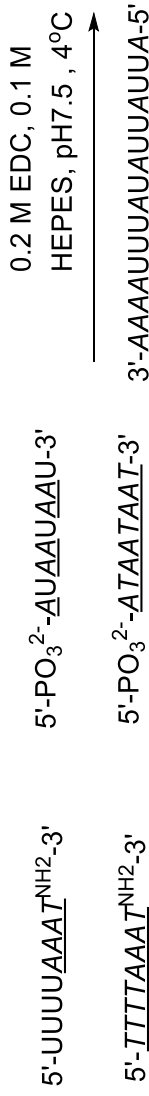
Entry	Sequence	Expected mass	Observed mass
Ligand R _{L3}	5'-UUUUAAA <u>T</u> ^{NH₂}	2453	2454
Ligand R _{L4}	5'-PO ₃ ²⁻ -AUAAUAAU-3'	2582	2583
Ligand C _{L3}	5'-UUUUAAA <u>T</u> ^{NH₂}	2405	2406
Ligand C _{L4}	5'-PO ₃ ²⁻ - <u>AUAAUAAU</u> -3'	2502	2503
Template C _{T2}	3'- <u>AAAAUUUAAUAAUUA</u> -5'	4893	4893
Homogeneous	5'-UUUUAAA <u>T</u> (NH)AUAUAAU-3'	5018	5017
Ligation product R _{P2}	1 h 4 h 24 h		
Yield (%)	43% 74% 92%		
Heterogeneous	5'-UUUUAAA <u>T</u> (NH) <u>AUAAUAAU</u> -3'	4890	4886
Ligation product C _{P2}	1 h 4 h 24 h		
Yield (%)	11% 22% 30%		

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 RDNA 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).

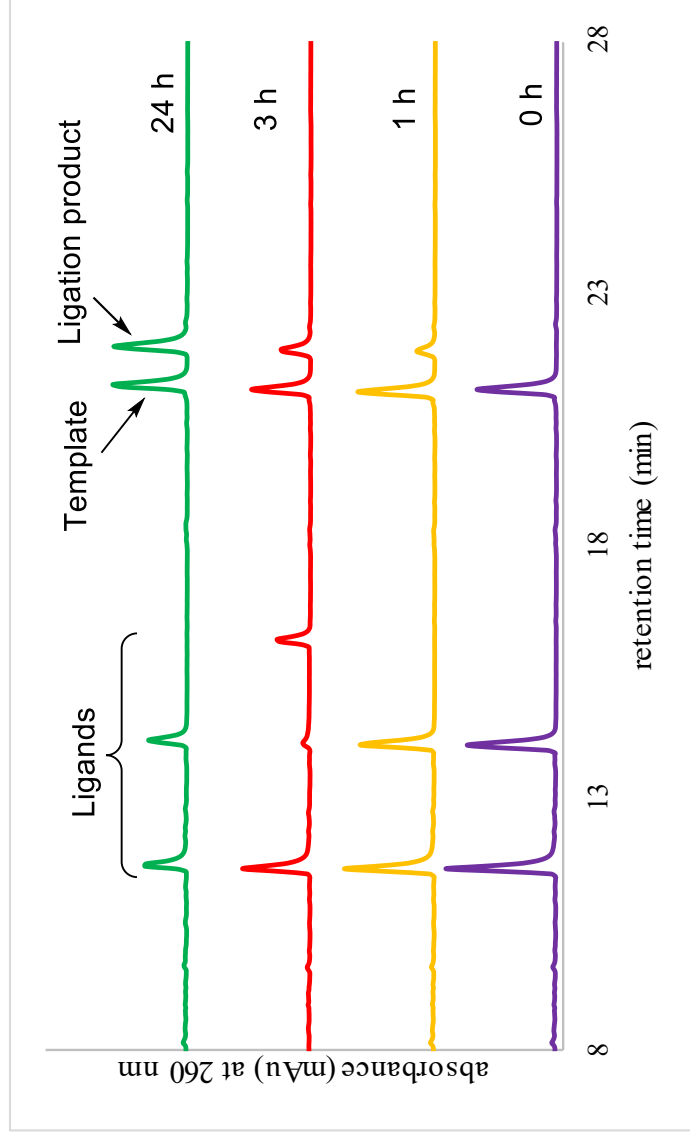
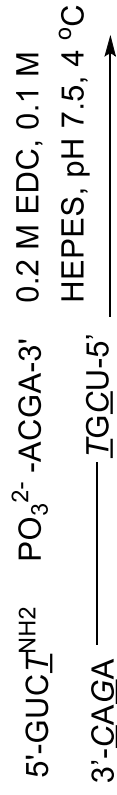
Competitive ligation reaction: RDNA chimeric ligands and DNA ligands on RDNA chimeric template



Entry	Sequence	Expected mass	Observed mass
Ligand DL1	5'- <u>TTTTAAAT</u> ^{NH2}	2397	2397
Ligand DL2	5'-PO ₃ ²⁻ - <u>ATAATAAT</u> -3'	2496	2495
Ligand CL3	5'-UUUU <u>AAAT</u> ^{NH2}	2405	2406
Ligand CL4	5'-PO ₃ ²⁻ - <u>AUAAUAAU</u> -3'	2502	2503
Template CT2	3'- <u>AAAAUUU</u> <u>AU</u> <u>AUU</u> <u>AUU</u> <u>A-5'</u>	4893	4893
Homogeneous	5'- <u>TTTTAAAT(NH)</u> <u>ATAATAAT</u> -3'	4876	4874
Ligation product DP1	1 h 4 h		
Yield (%)	23% 49%	78%	
Heterogeneous	5'-UUUU <u>AAAT(NH)</u> <u>AUAAUAAU</u> -3'	4890	Merged with the template peak
Ligation product CP2	1 h 4 h		
Yield (%)	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.*

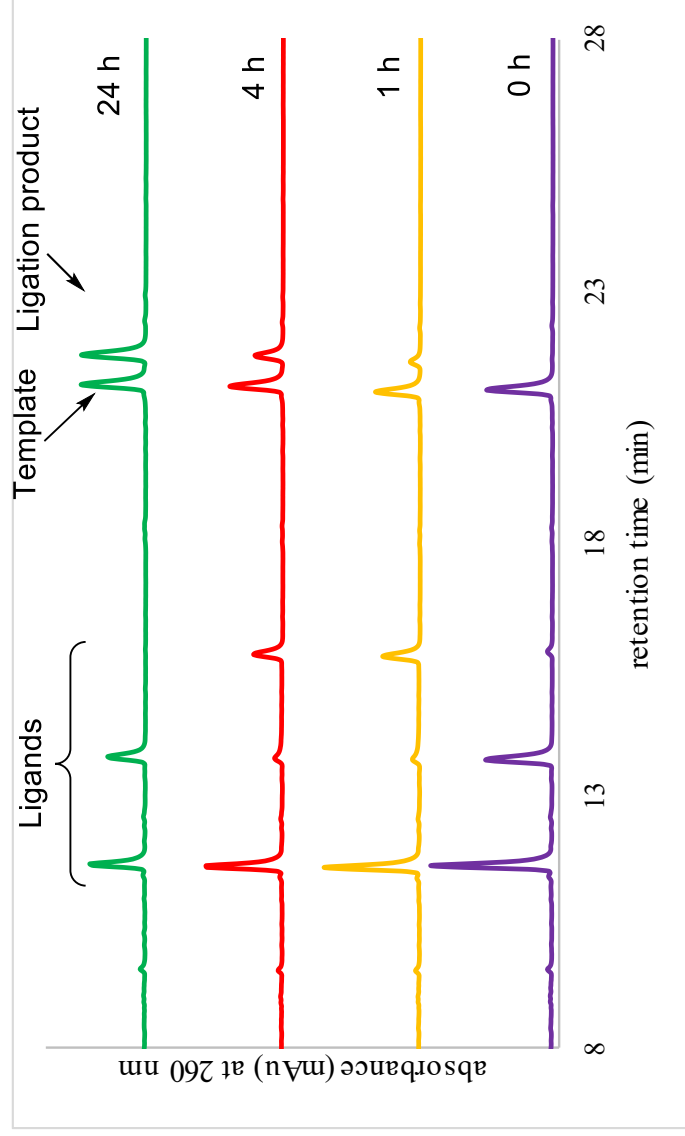
Ligation reaction. short tetrameric RNA ligands on octameric all four nucleobase RDNA chimeric template.



Entry	Sequence	Expected mass	Observed mass
Ligand R _{L7}	5'-GUC $\underline{T}^{\text{NH}_2}$	1199	1198
Ligand R _{L8}	5'-PO ₃ ²⁻ -ACGA-3'	1326	1327
Template C _{T4}	3'- $\underline{\text{CAGA}}$ $\underline{\text{TGCU}}$ -5'	2459	2460
Ligation product R _{P4}	5'-GTC $\underline{T}^{\text{(NH)}}$ ACGA-3'	2506	2508
Yield (%)	1 h	25%	
	3 h	55%	
	20 h		
		99%	

Supplementary Figure 50. AEC traces of the ligation reaction of short RNA 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.*

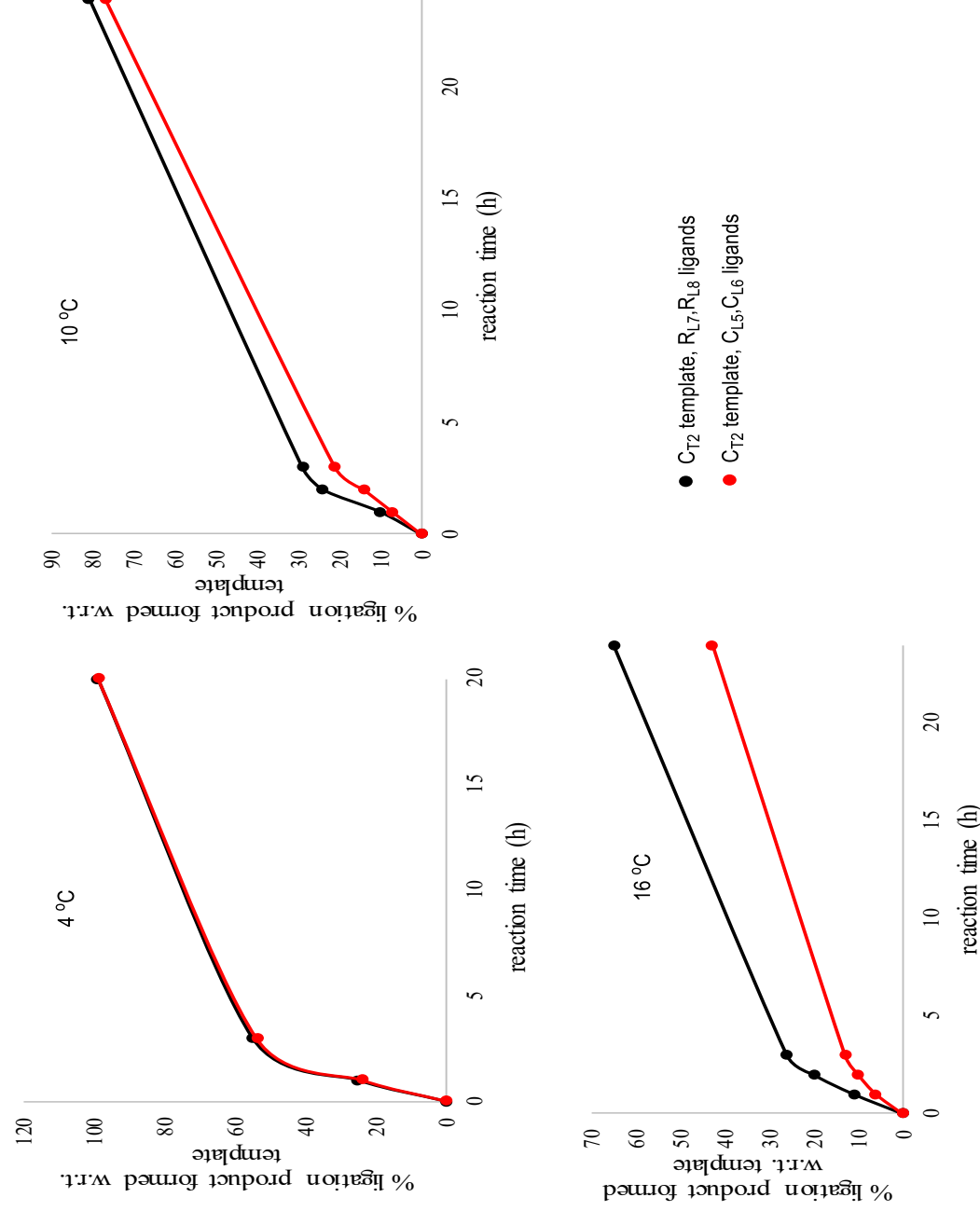
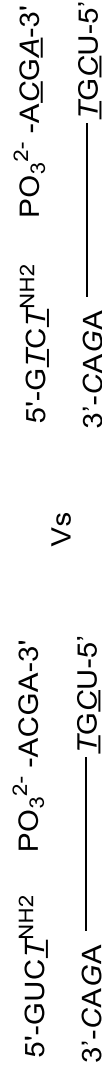
Ligation reaction. short tetrameric chimeric RDNA ligands on octameric all four nucleobase RDNA chimeric template.



Entry	Sequence	Expected mass	Observed mass
Ligand C _{L5}	5'-GTC ^{NH2}	1197	1197
Ligand C _{L6}	5'-PO ₃ ²⁻ -ACGA-3'	1294	1296
Template C _{T4}	3'-CAGATGCU-5'	2459	2461
Ligation	5'-GTC(NH)ACGA-3'	2472	2474
product C _{P3}	1 h 3 h 20 h		
Yield (%)	23% 53% 98%		

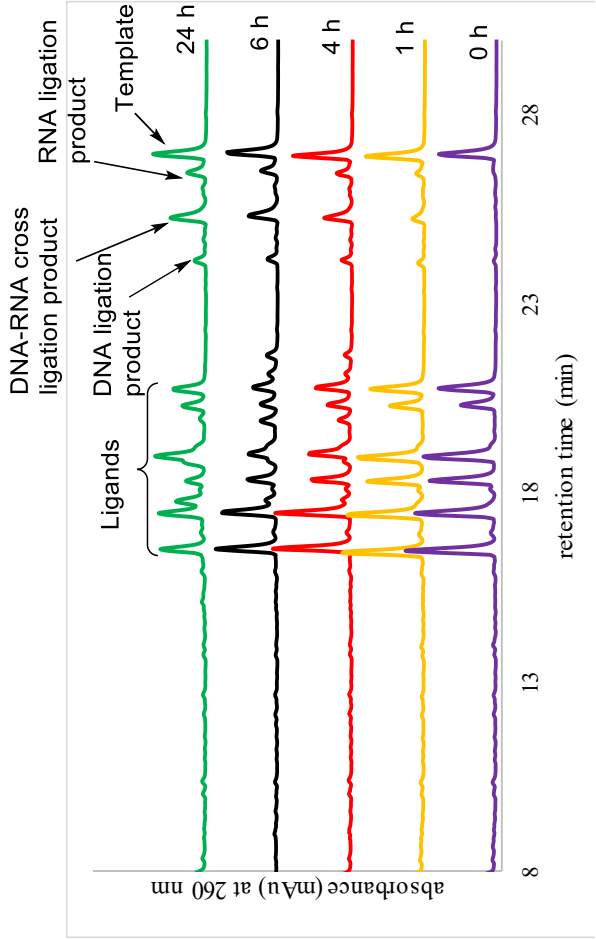
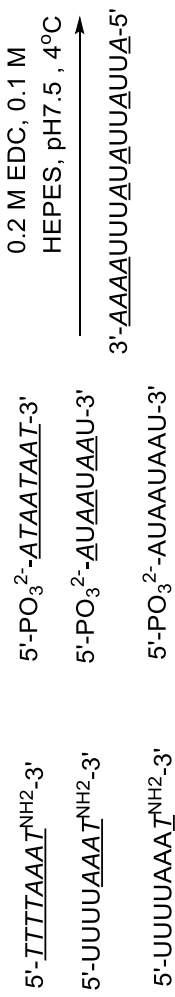
Supplementary Figure 51: AEC traces of the ligation reaction of short RDNA ligands (0.2 mM of each) on heterogeneous RDNA template (C_{T4}, 0.1 mM) with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The peak corresponding to the ligation product appeared at higher retention time than the template peak (Top 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



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.

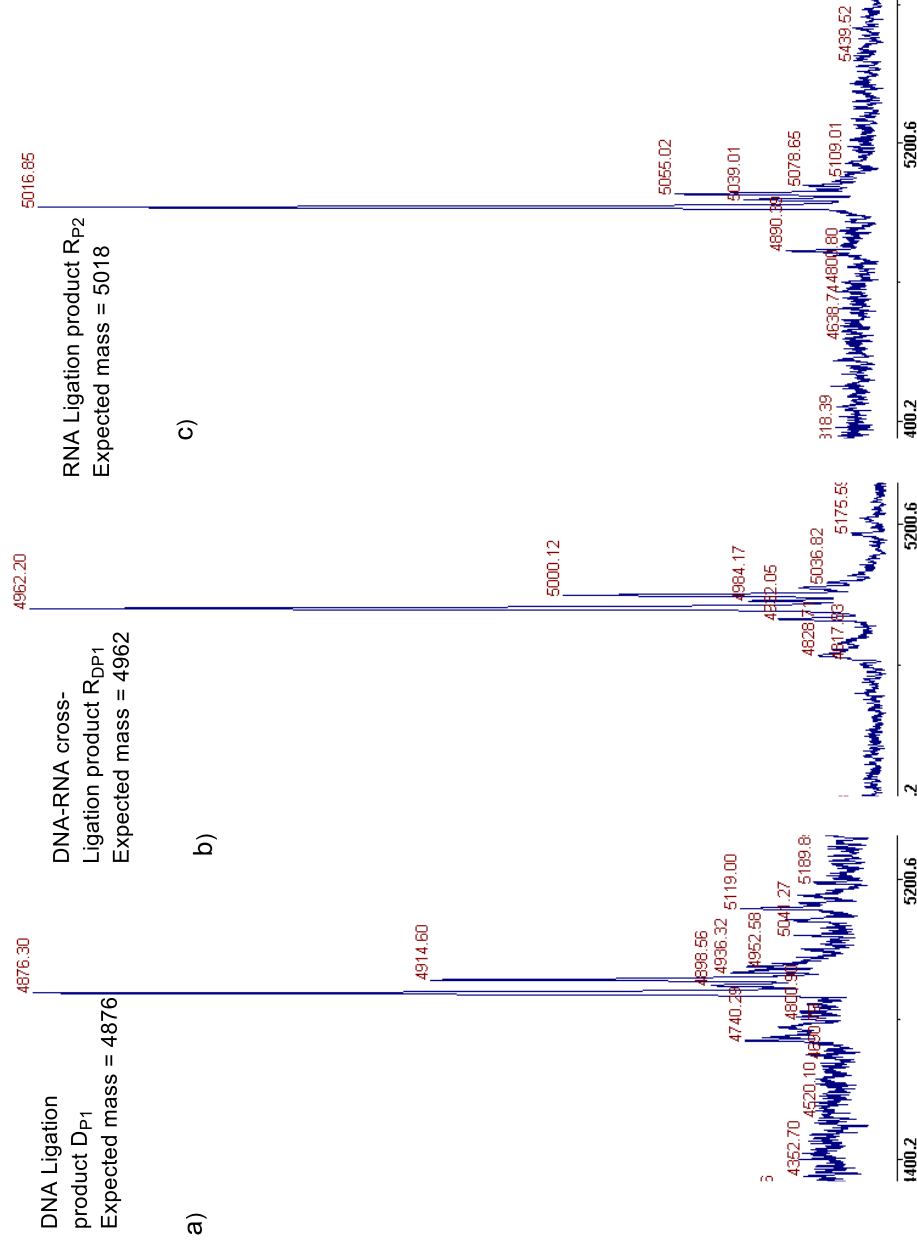
Competitive ligation reaction: RDNA chimeric ligands, DNA ligands and RNA ligands on RDNA chimeric template



Entry	Sequence	Expected mass	Observed mass
Ligand D _{L1}	5'- <u>TTTTT</u> AAAT ^{NH2}	2397	2398
Ligand D _{L2}	5'-PO ₃ ²⁻ - <u>ATAATAAT</u> -3'	2496	2497
Ligand C _{L3}	5'-UUUU <u>AAAT</u> ^{NH2}	2405	2406
Ligand C _{L4}	5'-PO ₃ ²⁻ - <u>AUAAUAAU</u> -3'	2502	2503
Ligand R _{L3}	5'-UUUUAAAA <u>T</u> ^{NH2}	2453	2453
Ligand R _{L4}	5'-PO ₃ ²⁻ -AUAAUAAU-3'	2582	2583
Template C _{T2}	3'- <u>AAAA</u> UUU <u>AUAAUAAU</u> A-5'	4893	4893
DNA Ligation product D _{P1}	5'- <u>TTTTT</u> AAAT(NH) <u>ATAATAAT</u> -3'	4876	4876
Yield (%)	1 h 10% 4 h 17% 24 h 20%		
RNA Ligation product R _{P2}	5'-UUUUAAAA <u>T</u> (NH)AUAAUAAU-3'	5018	5017
Yield (%)	1 h 20% 4 h 30% 24 h 38%		
Cross-Ligation product R _{DP1}	5'- <u>TTTTT</u> AAAT(NH) <u>AUAAUAAU</u> -3'	4962	4962
Yield (%)	1 h 27% 4 h 55% 24 h 75%		

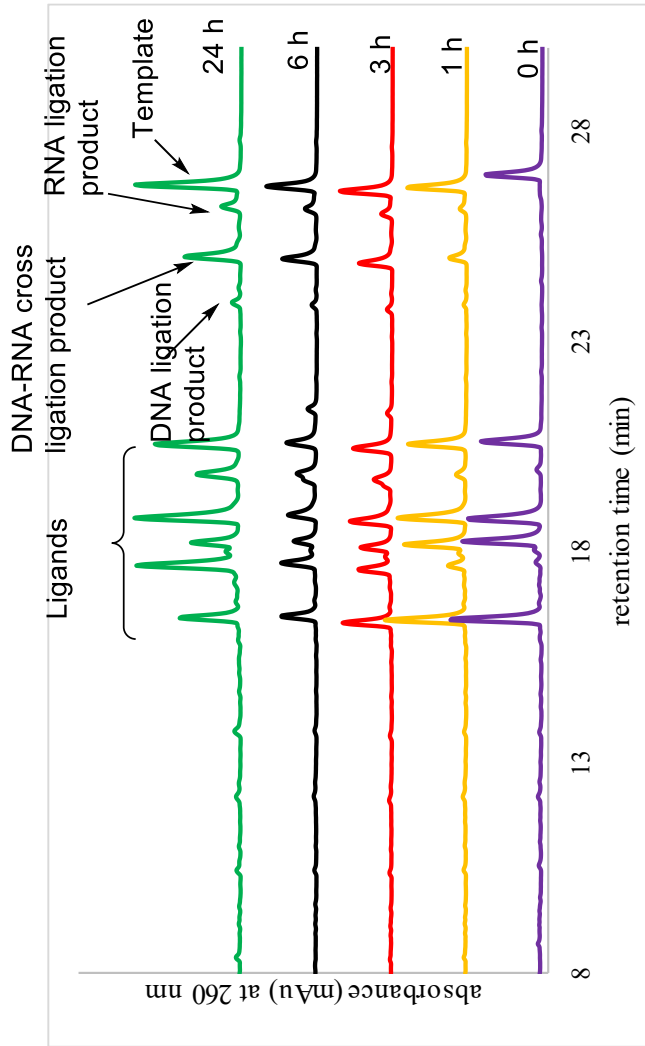
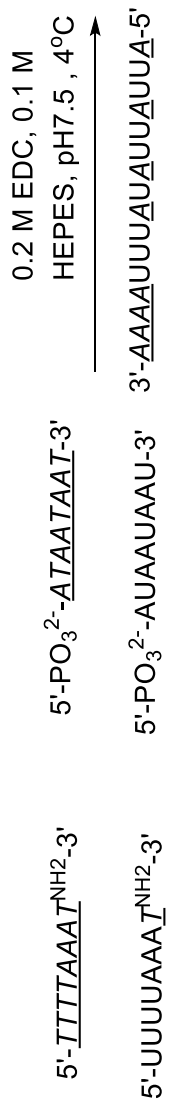
Supplementary Figure 53. AEC traces of the ligation reaction of RDNA chimeric ligands, DNA ligands and RNA ligands (0.2 mM of each) on RDNA chimeric template (0.1 mM) with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The AEC traces indicated the formation of three main peaks at lower retention time compared 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.*

MALDI-TOF-MS of the isolated peaks



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 Na_2HPO_4 , pH 11; Buffer B: 10 mM Na_2HPO_4 , 1 M NaCl, pH 11.

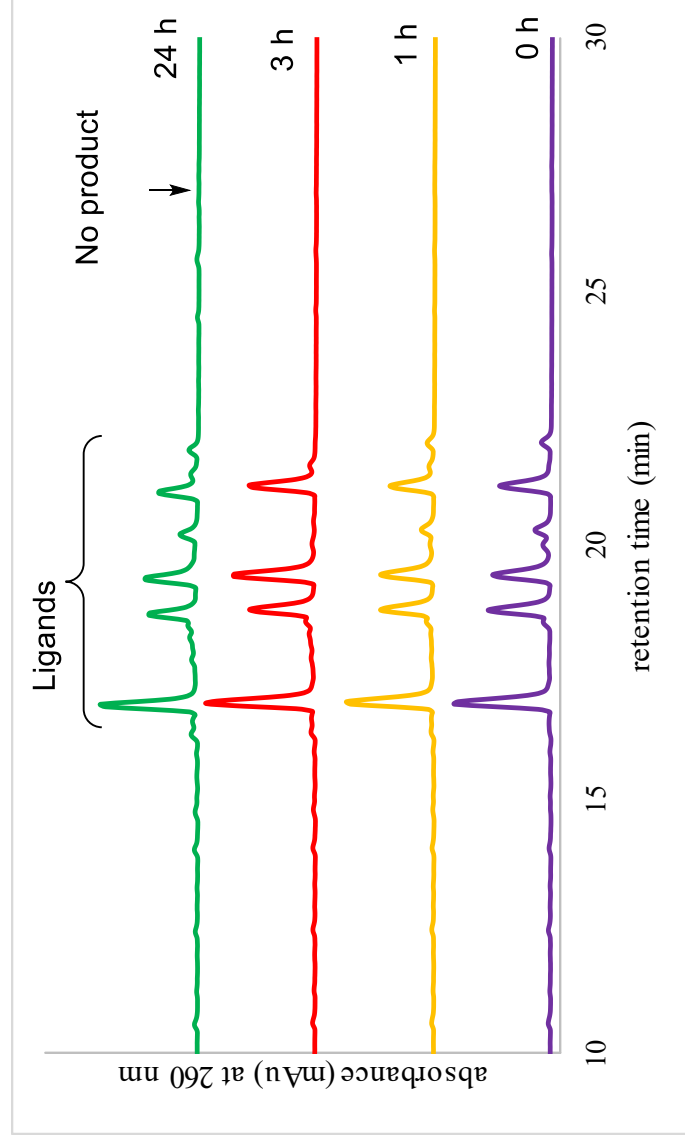
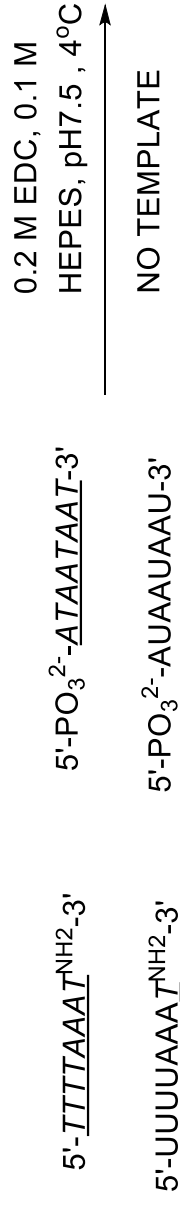
Competitive ligation reaction: DNA ligands and RNA ligands on RDNA chimeric template



Entry	Sequence	Expected mass	Observed mass
Ligand D _{L1}	5'- <u>TTTTT</u> AAA $\overline{T}^{NH_2}$	2397	2403
Ligand D _{L2}	5'-PO ₃ ²⁻ - <u>ATAATAAT</u> -3'	2496	2502
Ligand R _{L3}	5'-UUUUAAA $\overline{T}^{NH_2}$	2453	2459
Ligand R _{L4}	5'-PO ₃ ²⁻ -AUAUAUAU-3'	2582	2589
Template C _{T2}	3'- <u>AAAA</u> UUU <u>AU</u> <u>UUU</u> <u>UUU</u> <u>A-5'</u>	4893	4900
DNA Ligation product D _{P1}	5'- <u>TTTTT</u> AAA $\overline{T}^{NH_2}$ (NH) <u>ATAATAAT</u> -3'	4876	4883
Yield (%)	1 h 4 h 24 h 7% 12% 14%		
RNA Ligation product R _{P2}	5'-UUUUAAA $\overline{T}^{NH_2}$ (NH)AUAUAUAU-3'	5018	5026
Yield (%)	1 h 4 h 24 h 16% 25% 31%		
Cross-Ligation product R _{DP1}	<u>5'-TTTTT</u> AAA $\overline{T}^{NH_2}$ (NH)AUAUAUAU-3'	4962	4969
Yield (%)	1 h 4 h 24 h 33% 67% 72%		

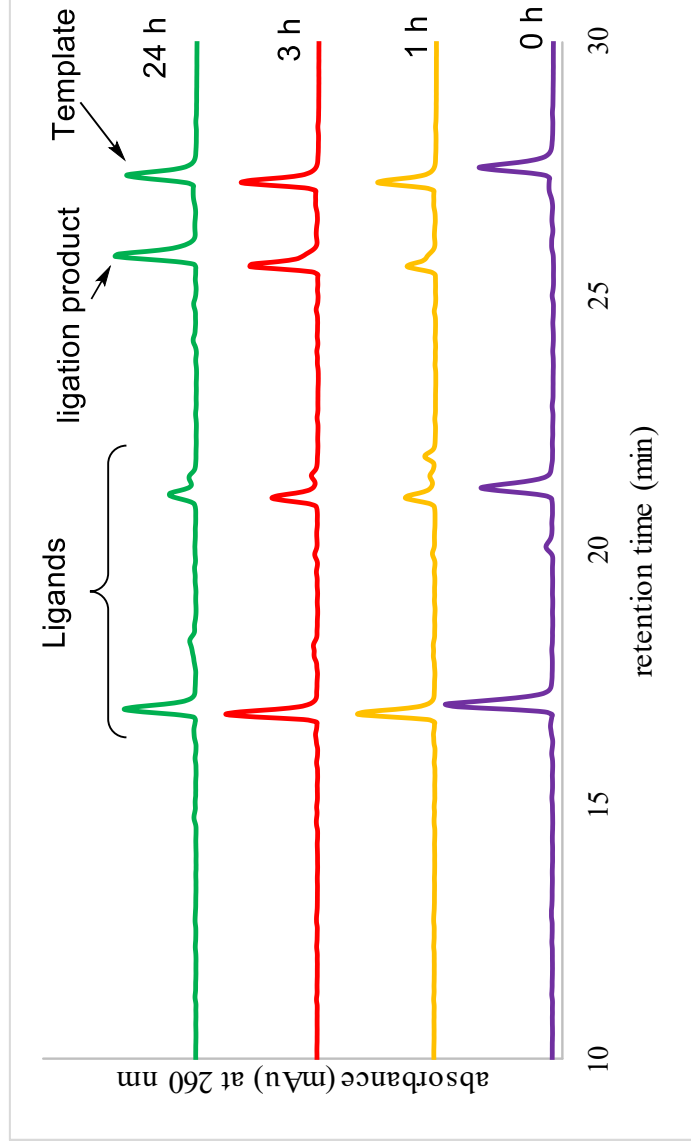
Supplementary Figure 55. AEC traces of the ligation reaction of DNA ligands and RNA ligands (0.2 mM of each) on RDNA chimeric template (0.1 mM) with 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The AEC traces indicate the formation of DNA ligation product (14%), RDNA cross-ligation product (72%) and RNA ligation product (31%). *Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.*

Control reaction: parallel DNA ligands (D_{L1}, D_{L2}) and RNA ligands (R_{L3}, R_{L4}) in absence of any template



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.*

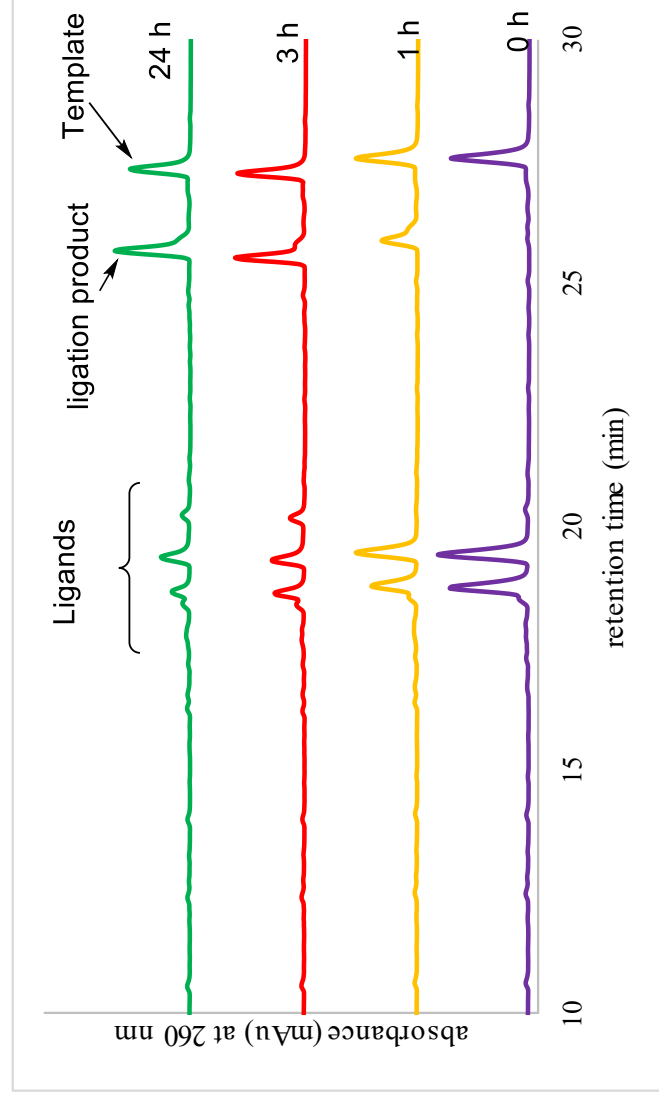
Cross-ligation reaction: RNA $\underline{T}^{\text{NH}_2}$ (R L_3) and PO $_3^{2-}$ -DNA (D L_2) ligands on RDNA chimeric template (C T_2)



Entry	Sequence	Expected mass	Observed mass
Ligand R L_3	5'-UUUUAAA $\underline{T}^{\text{NH}_2}$	2453	2453
Ligand D L_2	5'-PO $_3^{2-}$ - $\underline{ATAATAAT}$ -3'	2496	2496
Template C T_2	3'- $\underline{AAAA}$ UUU <u>AU</u> UU <u>AUUA</u> -5'	4893	4893
Cross-Ligation product R DP_2	5'-UUUUAAA <u>T(NH)</u> <u>ATAATAAT</u> -3'	4932	4930
Yield (%)	1 h	4 h	24 h
	60%	99%	119%

Supplementary Figure 57. AEC traces of the ligation reaction of RNA $\underline{T}^{\text{NH}_2}$ (R L_3) ligands and PO $_3^{2-}$ -DNA (D L_2) 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 DP_2 (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.*

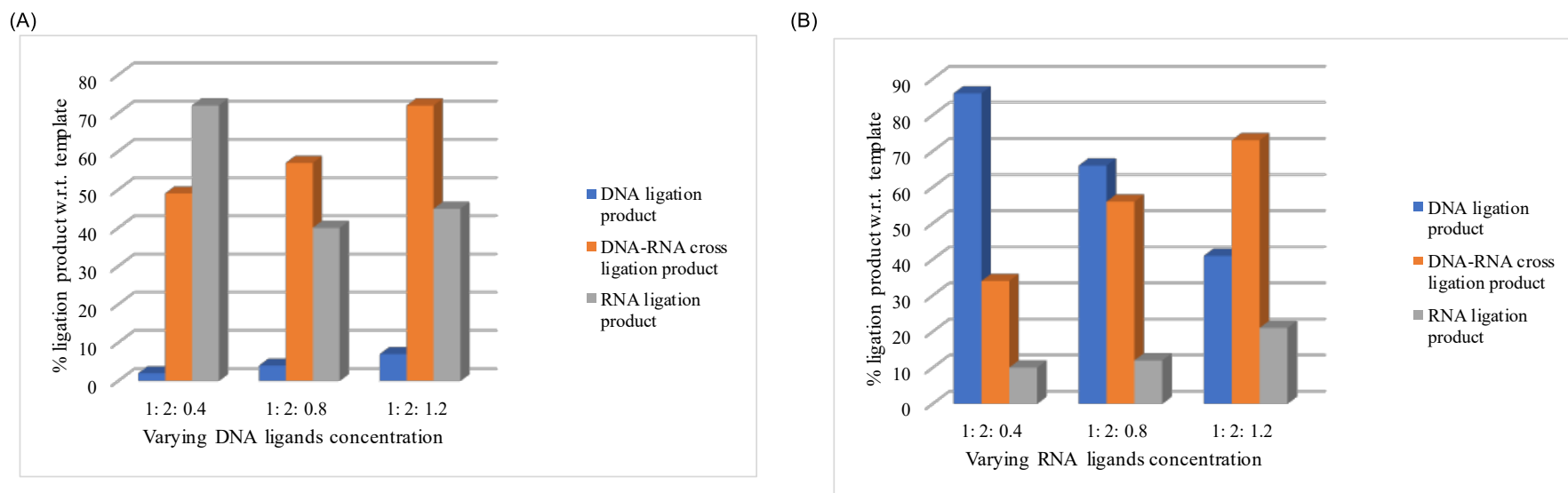
Cross-ligation reaction: DNA^{NH₂} (D_{L1}) and PO₃²⁻-RNA (R_{L4}) ligands on RDNA chimeric template (C_{T2})



Entry	Sequence	Expected mass	Observed mass
Ligand D _{L1}	5'- <u>TTTTAAAT</u> ^{NH₂}	2397	2398
Ligand R _{L4}	5'-PO ₃ ²⁻ -AUAAUAAU-3'	2582	2583
Template C _{T2}	3'- <u>AAAAUUUAU</u> <u>AUUAUUA</u> -5'	4893	4893
Cross-Ligation product R _{DP1}	5'- <u>TTTTAAAT</u> (NH) <u>AUAAUAAU</u> -3'	4962	4962
Yield (%)	1 h	4 h	24 h
	68%	114%	131%

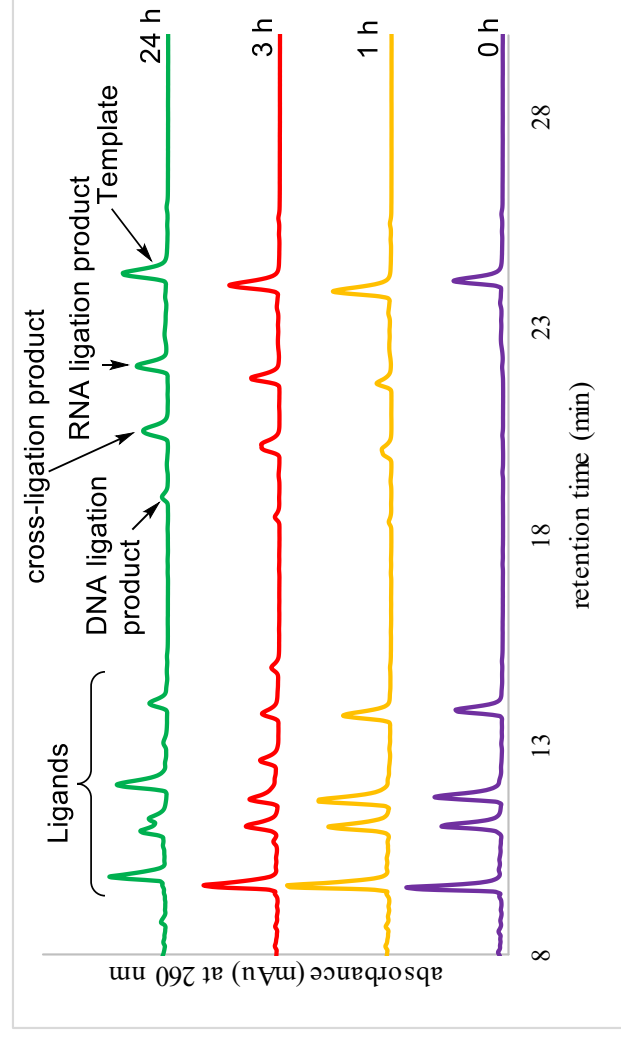
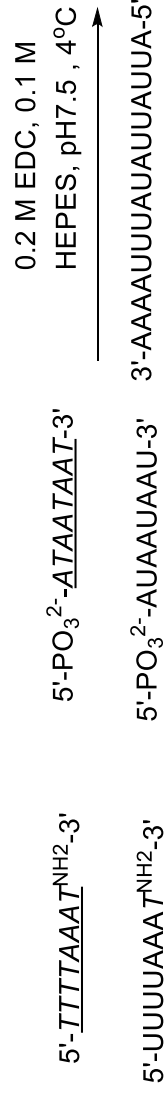
Supplementary Figure 58. AEC traces of the ligation reaction of DNA^{NH₂} (D_{L1}) ligand and PO₃²⁻-RNA (R_{L4}) ligands on RDNA chimeric template in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer with 0.1 mM template and 0.2 mM of each ligands. MALDI of the 24 h reaction mixture confirms the formation of cross-ligation 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 $\pm 5\%$.

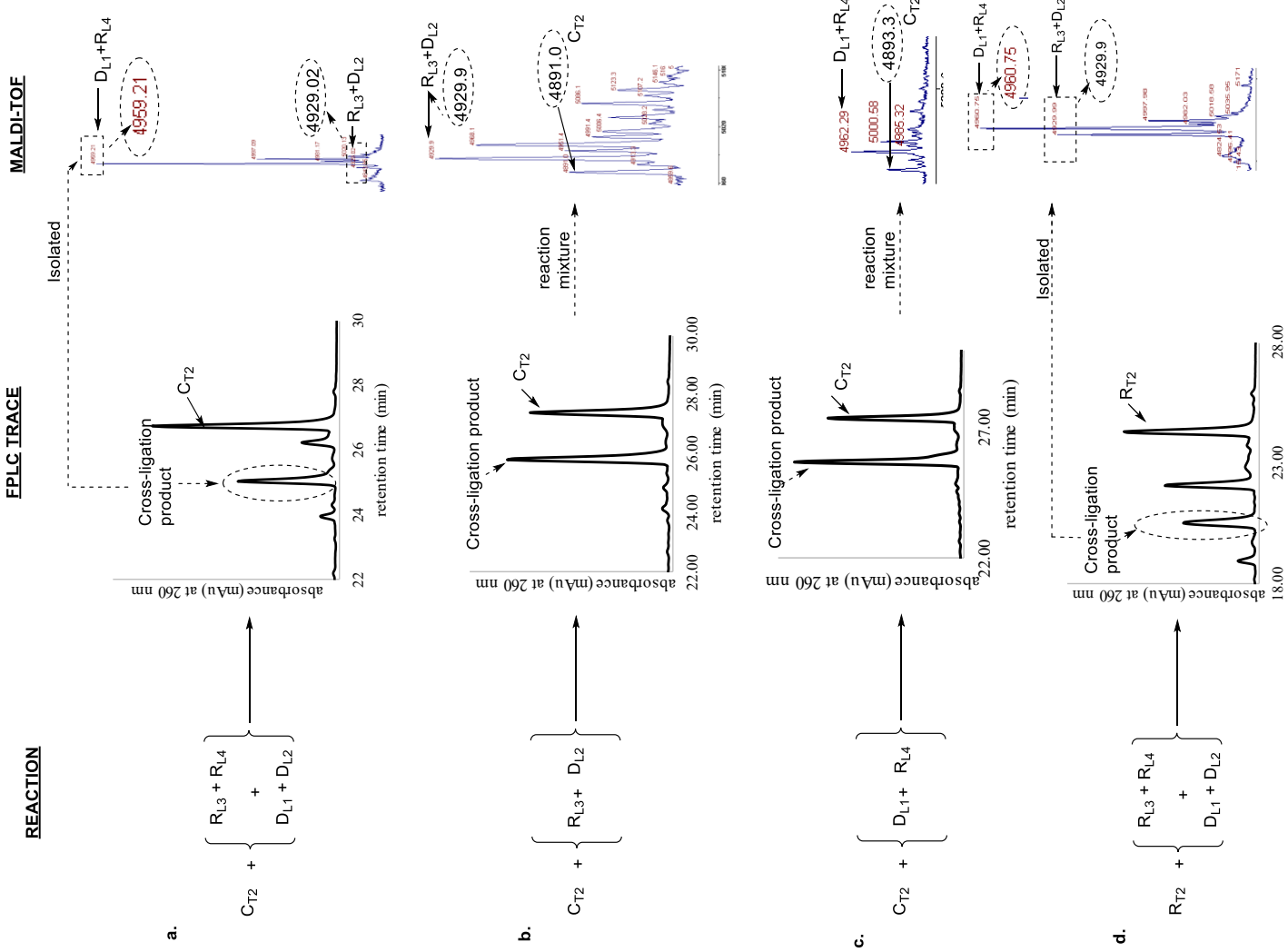
Competitive ligation reaction: DNA ligands and RNA ligands on RNA template



Entry	Sequence	Expected mass	Observed mass
DNA Ligation product DP ₁	5'- <u>TTTTAAAT</u> (NH) <u>ATAATAAT</u> -3'	4876	4876
Yield (%)	1 h 4% 4 h 6% 24 h 12%		
RNA Ligation product RP ₂	5'-UUUUAAA <u>T</u> (NH)AUAAUAAU-3'	5018	5016
Yield (%)	1 h 22% 4 h 51% 24 h 65%		
Cross-Ligation product R _{DP1} + R _{DP2}	5'- <u>TTTTAAAT</u> (NH)AUAAUAAU-3' + 5'-UUUUAAA <u>T</u> (NH) <u>ATAATAAT</u> -3'	4962	4961
Yield (%)	1 h 21% 4 h 45% 24 h 62%	4932	4930

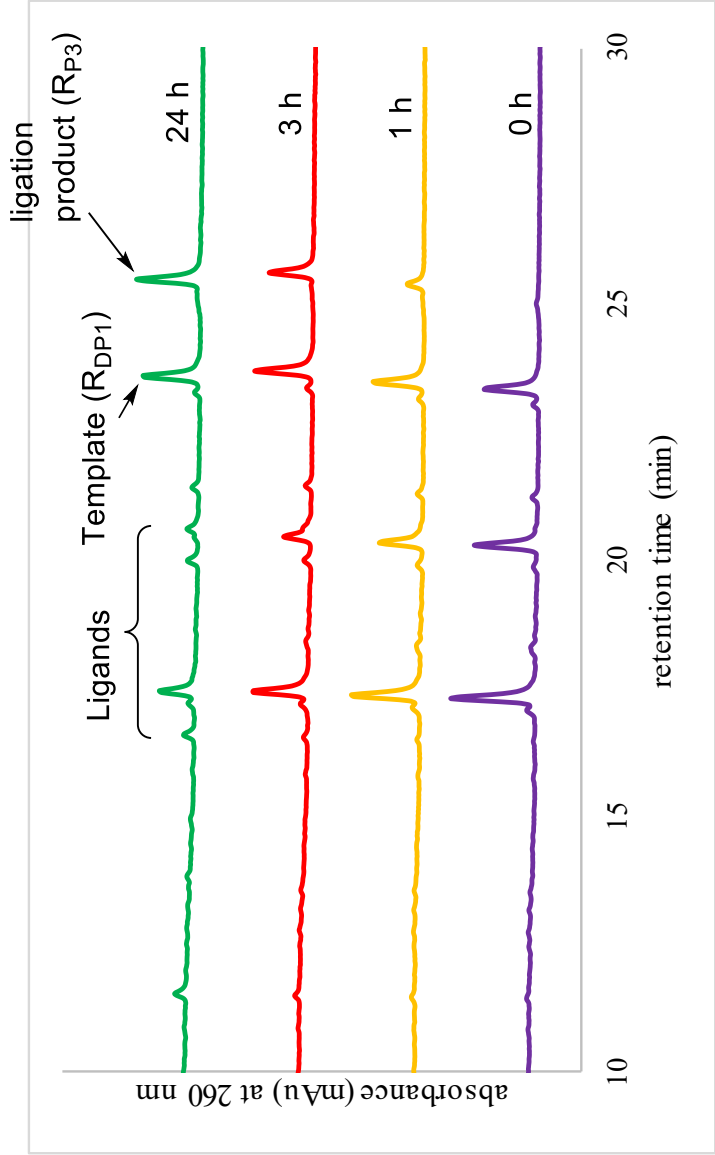
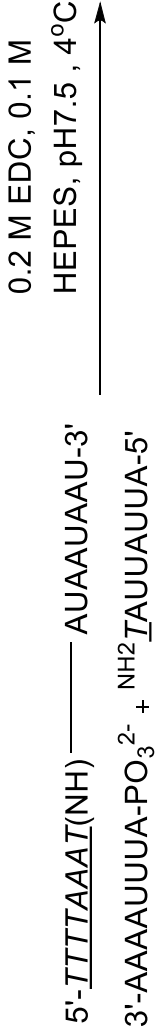
Supplementary Figure 60. AEC traces of the ligation reaction of DNA ligands and RNA ligands on RNA template in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer with 0.1 mM template and 0.2 mM of each ligands. The AEC traces indicate the formation of DNA ligation product (12%), RDNA cross-ligation product (62%) and 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 $R_{L3}, R_{L4}, D_{L1}, D_{L2}$ on C_{T2} template and MALDI-TOF of the isolated cross-ligation peak indicate it to be R_{DP1} as major. **Figure b.** Reaction of R_{L3} and D_{L2} on C_{T2} template and MALDI-TOF of the 24 h reaction mixture indicate it to be R_{DP2} . **Figure c.** reaction of D_{L1} and R_{L4} on C_{T2} template and MALDI-TOF of the 24 h reaction mixture indicates it to be R_{DP1} . **Figure d.** Reaction of $R_{L3}, R_{L4}, D_{L1}, D_{L2}$ on R_{T2} template and MALDI-TOF of the isolated cross-ligation peak indicate it to be the mixture of R_{DP1} and R_{DP2} of close intensity. Both the R_{DP1} and R_{DP2} appear at the same retention time in AEC as observed in the spiking experiment.

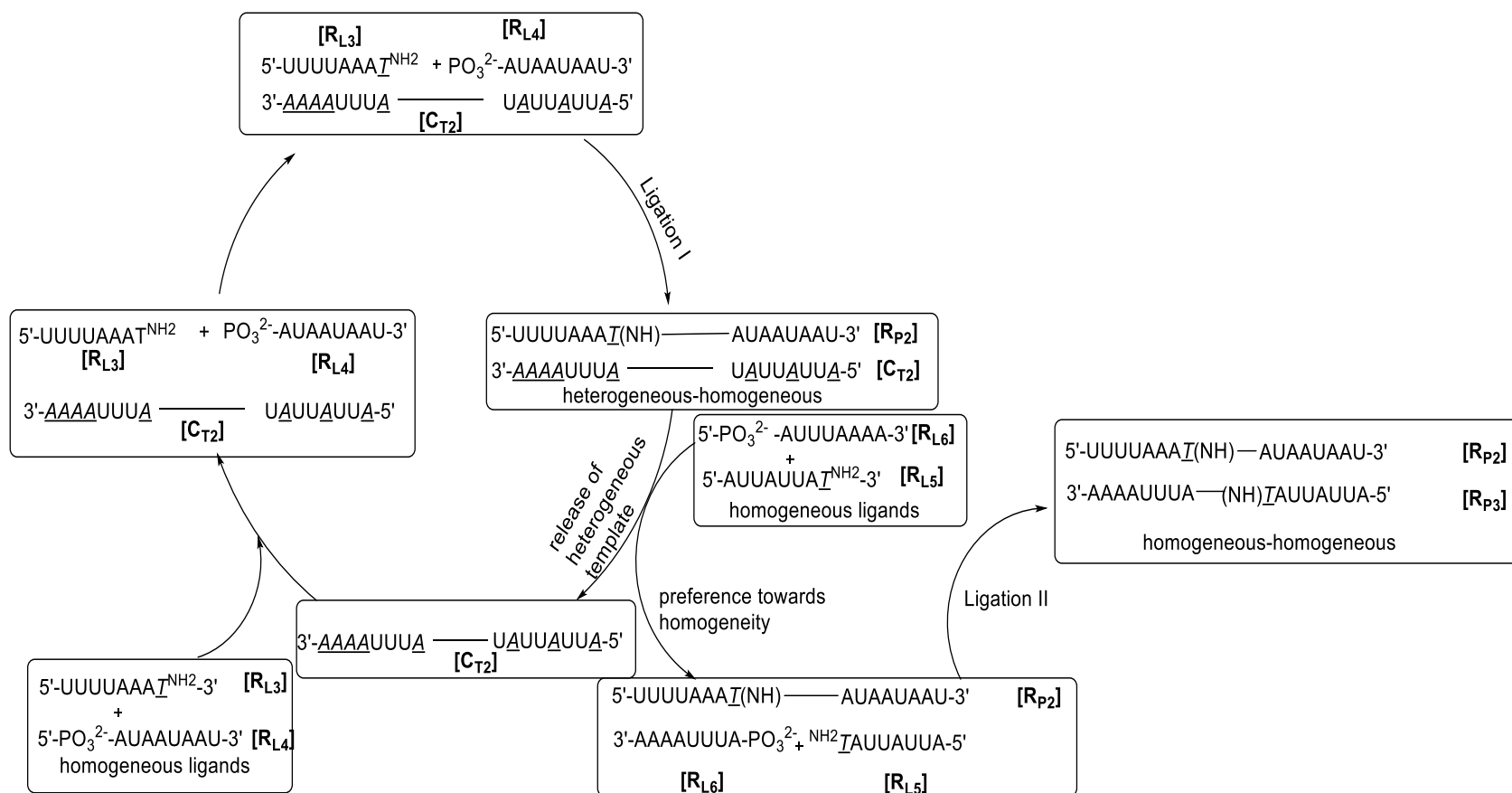
Cross-ligation product (RD_{P1}) as template in the ligation reaction



Entry	Sequence	Expected mass	Observed mass
Ligand R _{L6}	5'-PO ₃ ²⁻ -AUUUAAAA-3'	2582	2582
Ligand R _{L5}	5'-AUUAUUAT ^{NH2} -3'	2453	2453
Template RD _{P1}	5'- <u>TTTTAAAT</u> (NH)AUAUAUU-3'	4962	4959
Ligation product R _{P3}	3'-AAAAUUUA <u>T</u> (NH)AUUAUUA-5'	5018	5015
Yield (%)		33% 4 h 73% 24 h 108%	

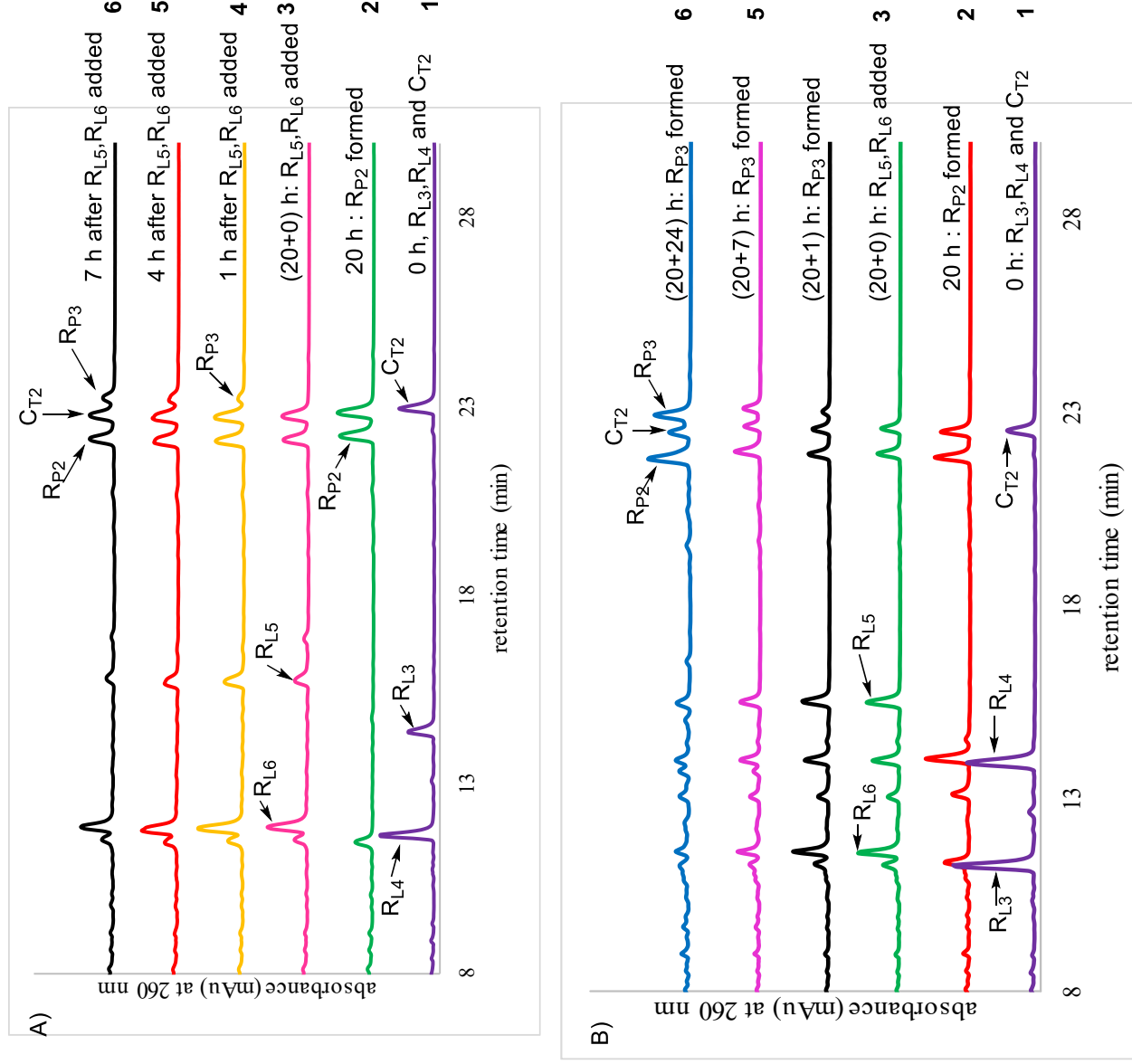
Supplementary Figure 62. AEC traces of the ligation reaction of RNA ligands (0.2 mM each) (R_{L5},R_{L6}) on cross-ligation product template R_{DP1} (0.1 mM) in 0.2M EDC, 0.1M HEPES, pH 7.5 buffer with 0.1 mM template and 0.2 mM of each ligands. *Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.*

Stepwise replication cycle. RNA ligands on RDNA chimeric template



Supplementary Figure 63. The expected DNA-RNA chimeric-system-based replicating cycle. It was anticipated that the addition of ligands (R_{L5}, R_{L6}), complementary to the 1st ligation product (R_{P2}) will release the chimeric template (C_{T2}) due to the preference of homogeneous ligands for homogeneous template and the released chimeric template will continue the replication-by-ligation further.

Stepwise replication cycle. AEC traces of the stepwise replication cycle of RNA ligands on RDNA chimeric template

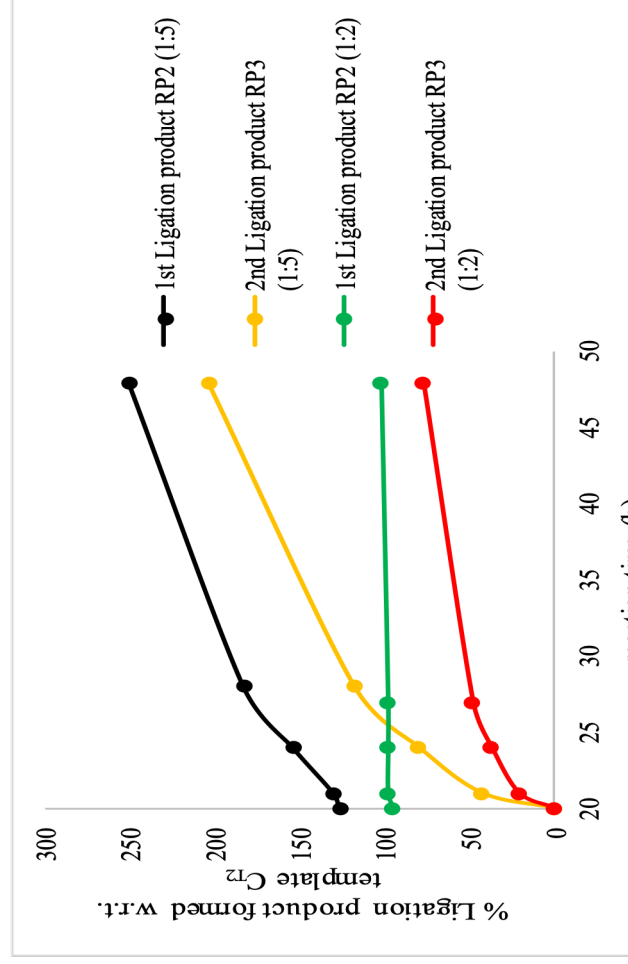


Supplementary Figure 64. AEC traces of the DNA-RNA chimeric system based self-replicating system using *in situ* generated ligation product (R_{P2}) as template for second ligation reaction. Figure (A) The reaction of R_{L3}, R_{L4} (0.2 mM of each ligands) on DNA-RNA chimeric template C_{T2} (0.1 mM) produces the 1st ligation product (R_{P2}) (bottom two spectra). Addition of ligands R_{L5}, R_{L6} (0.42 mM of each in 2 μ l new buffer) causes the second ligation reaction to form 2nd ligation product R_{P3} (spectra 3-6, Top trace). During that reaction the initial ratio of C_{T2} : R_{L3}, R_{L4} was (1:2) and after R_{L5}, R_{L6} addition the final ratio of C_{T2} : R_{L5}, R_{L6} became (1:2:2). Figure (B) The reaction of R_{L3}, R_{L4} (0.5 mM of each ligands) on DNA-RNA chimeric template C_{T2} (0.1 mM) produces the 1st ligation product (R_{P2}) (bottom two spectra). Addition of ligands R_{L5}, R_{L6} (0.42 mM in 2 μ l new buffer) causes the second ligation reaction to form 2nd ligation product R_{P3} (spectra 3'-6', Bottom spectra). During that reaction the initial ratio of C_{T2} : R_{L3}, R_{L4} was (1:5) and after R_{L5}, R_{L6} addition the final ratio of C_{T2} : R_{L3}, R_{L4} : R_{L5}, R_{L6} became (1:5:2).

Stepwise replication cycle. % 1st and 2nd ligation product formation in replication cycle of RNA ligands on RDNA chimeric template

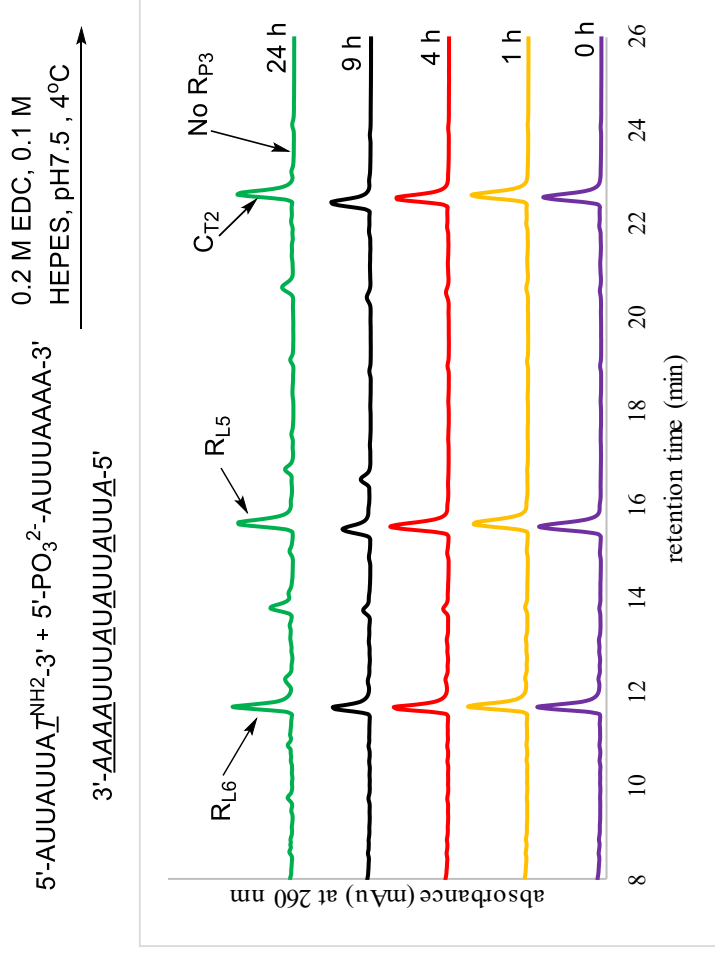
Reaction time (h)	Initial Template (C _{T2}):Ligands (R _{L3} ,R _{L4}) (1:2)		Initial Template (C _{T2}):Ligands (R _{L3} ,R _{L4}) (1:5)	
	% 1 st ligation product (R _{P2})	% 2 nd ligation product (R _{P3})	% 1 st ligation product (R _{P2})	% 2 nd ligation product (R _{P3})
0	0	-	0	-
20	94	-	126	-
(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 (C_{T2}) 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 1st and second ligation product. Although the R_{L5},R_{L6} concentration remain same but the presence of higher concentration of R_{L3},R_{L4} (1:5 Vs 1:2) increases the formation of R_{P3} (204% Vs 77%, bottom entry). The bottom Figure is the graphical presentation of the formation of R_{P2} and R_{P3} with different initial concentration of R_{L3},R_{L4} (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 $\pm 5\%$.

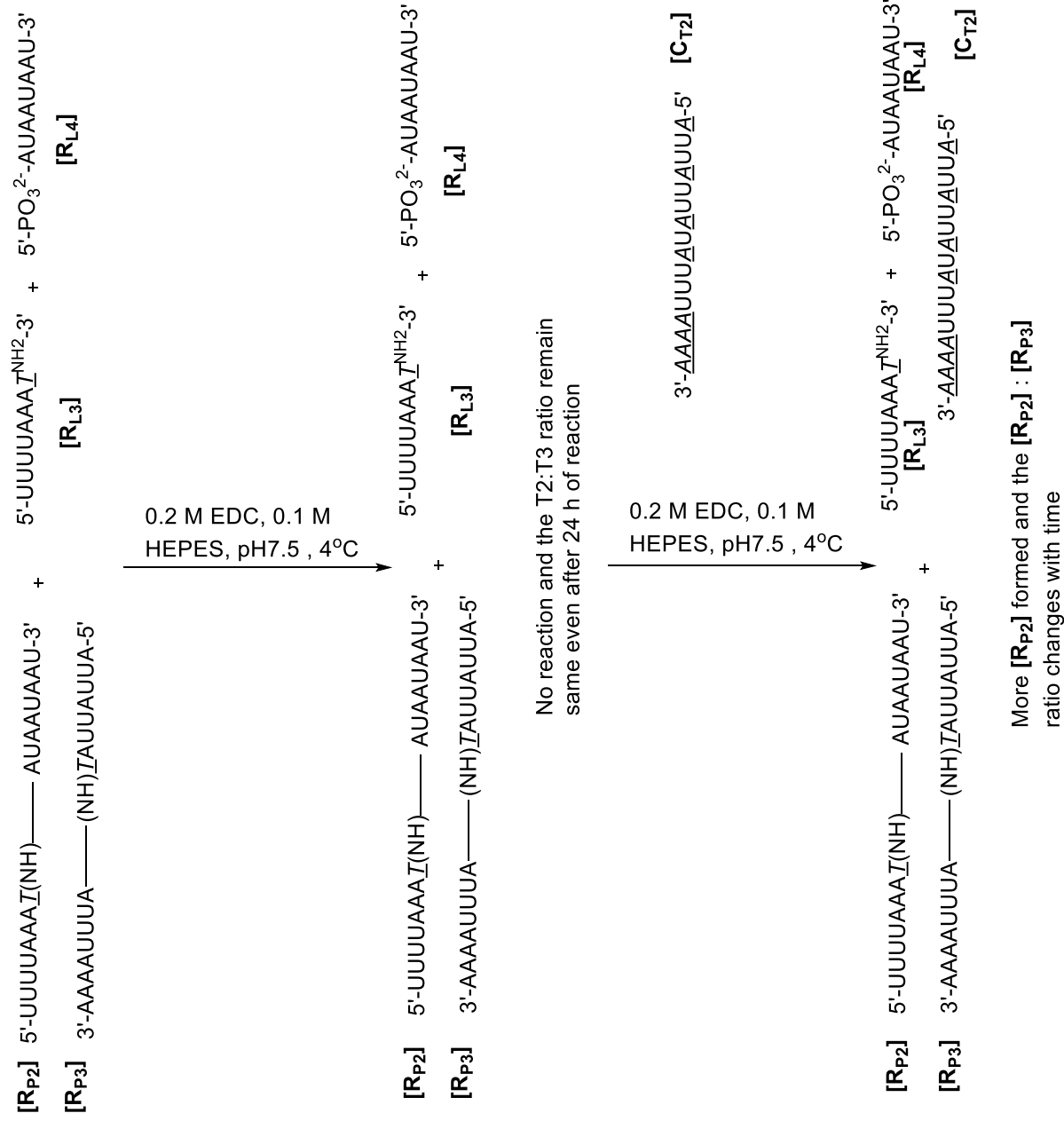
Control ligation reaction. Chimeric RDNA (C_{T2}) template and its parallel RNA (R_{L5} , R_{L6}) ligands



Entry	Sequence	Expected mass	Observed mass
Ligand R_{L5}	$5'\text{-PO}_3^{2-}\text{-AUUUUAAAA-}3'$	2582	2582
Ligand R_{L6}	$5'\text{-AUUAUUUA}\underline{T}^{\text{NH}_2}\text{-}3'$	2453	2452
Template C_{T2}	$3'\text{-}\underline{AAAA}\text{UUUA}\underline{AU}\underline{AU}\underline{AU}\underline{AU}\underline{A-}5'$	4893	4890
Ligation product R_{P3}	$5'\text{-AUUUAAAA(NH)}\underline{T}\text{AUUAUUUA-}3'$	5018	Not observed

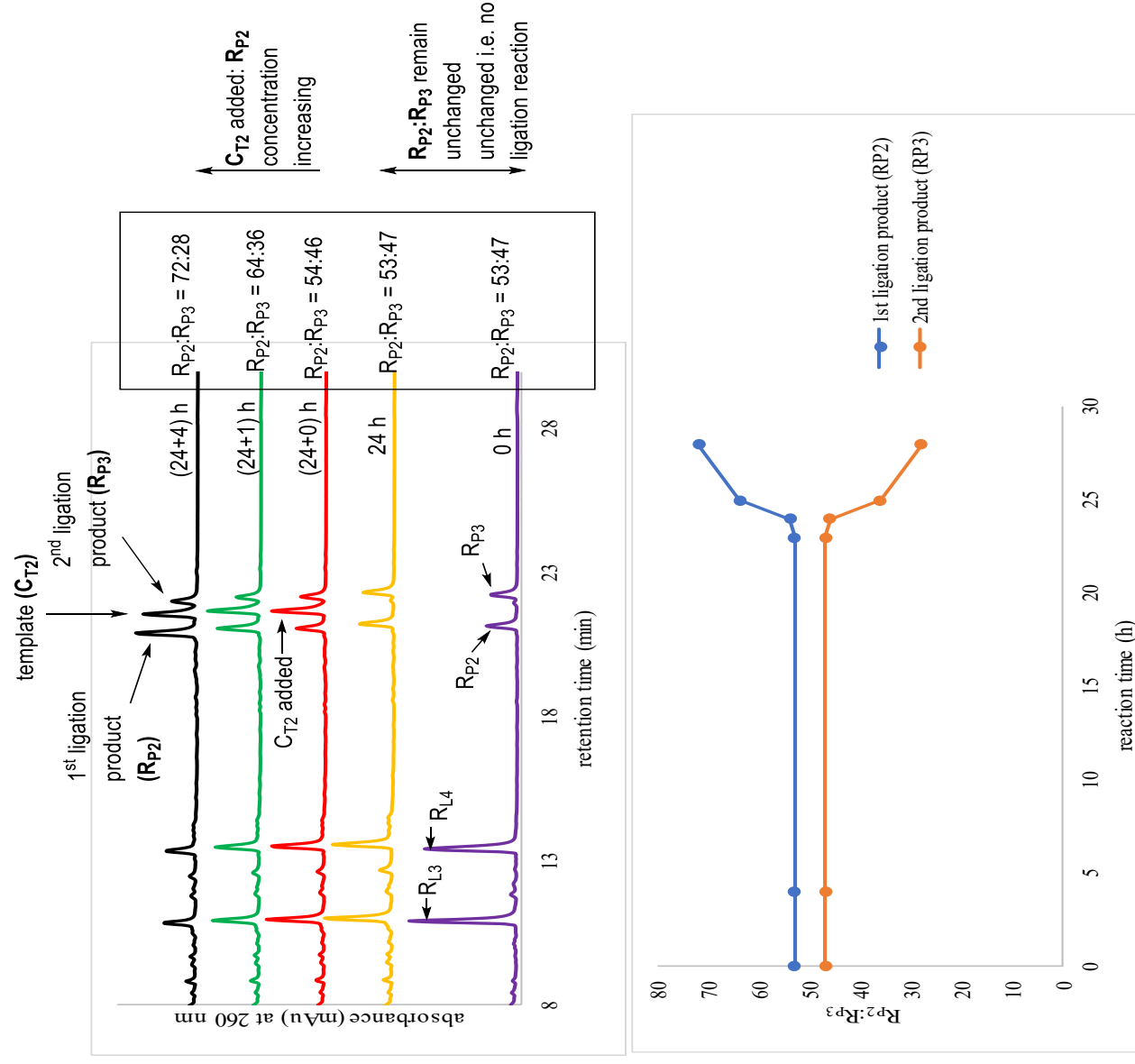
Supplementary Figure 66. The AEC traces of the ligation reaction of homogeneous RNA bottom strand ligands R_{L5} , R_{L6} (0.2 mM each) in presence of DNA-RNA chimeric template C_{T2} (0.1 mM) in 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. C_{T2} is parallel to R_{L5} , R_{L6} and the AEC traces indicates that the no R_{P3} 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.



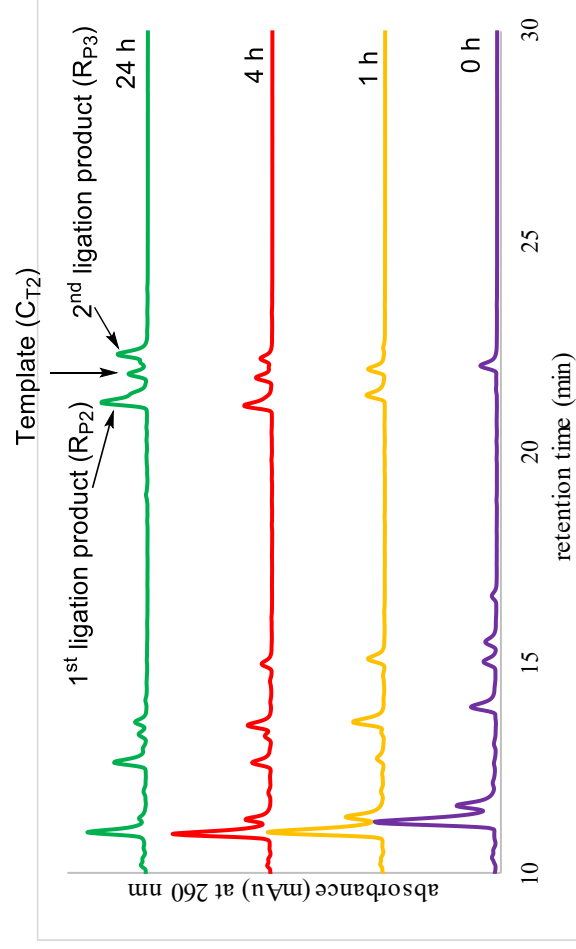
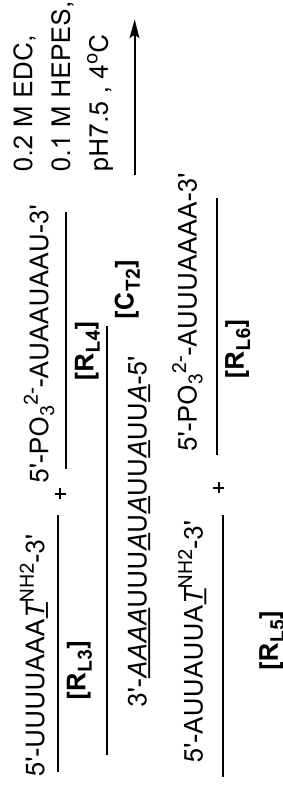
Supplementary Figure 67. The ligation reaction of RNA (R_{L3}, R_{L4}) ligands (0.2 mM each) in the presence of two complementary ligation (R_{P2}, R_{P3}) products (0.1 mM each) in 0.2 M EDC, 0.1 M HEPES, pH 7.5 buffer. After, 24 h of reaction RDNA chimeric (C_{T2}) template was added to the reaction 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_{T2}) in the continuous production of 1st ligation product (R_{P2}) in the presence of two complementary homogeneous ligation products R_{P2} and R_{P3} . The AEC traces indicate the ligation reaction of R_{L3} , R_{L4} (0.2 mM of each) in presence of R_{P2} and R_{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_{P2} and R_{P3} indicate that no ligation reaction occur even after 24 h of reaction (bottom two spectra). Then the addition of C_{T2} changes the R_{P2} and R_{P3} ratio indicating the ligation reaction. Bottom Figure indicated the change in R_{P2} and R_{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.

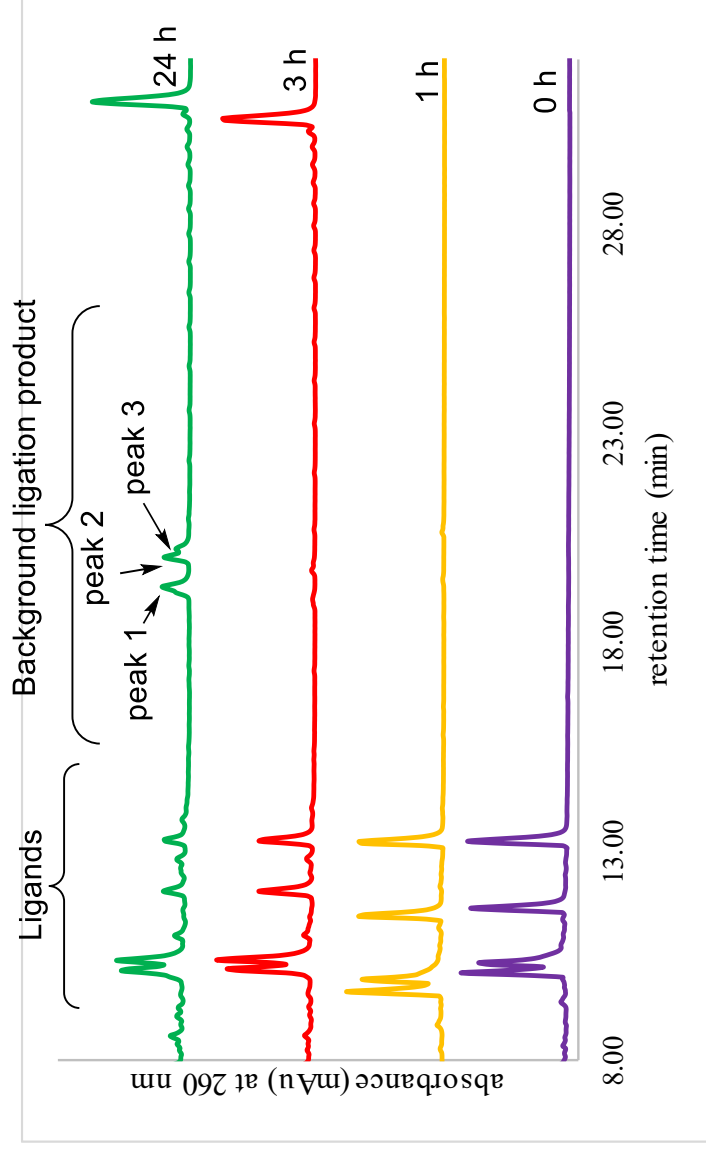
One-pot replication cycle. All four RNA ligands on RDNA chimeric template



Entry	Sequence
Ligand R _{L3}	5'-UUUUAAA <u>T</u> ^{NH₂} -3'
Ligand R _{L4}	5'-PO ₃ ²⁻ -AUAAUAAU-3'
Ligand R _{L6}	5'-PO ₃ ²⁻ -AUUUUAAAA-3'
Ligand R _{L5}	5'-AUUAAUU <u>A</u> ^{NH₂} -3'
Template C _{T2}	3'- <u>AAAA</u> UUUU <u>AUAAUAAU</u> <u>A</u> -5'
1 st Ligation product R _{P2}	5'-AUUUUAAAA(NH) <u>TAUUAUU</u> A-3'
Yield (%)	1 h 4 h 24 h
	113% 201% 259%
2 nd Ligation product R _{P3}	3'-AAAAUUUUA(NH) <u>TAUUAUU</u> A-5'
Yield (%)	1 h 4 h 24 h
	9% 76% 191%

Supplementary Figure 69: The AEC traces of the one-pot ligation reaction of R_{L3}, R_{L4} (0.5 mM of each) R_{L5}, R_{L6} (0.2 mM of each) on DNA-RNA chimeric template C_{T2} (0.1 mM) in 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The AEC traces indicates the appearance of 1st ligation product (R_{P2}) in lower retention time than the template and 2nd ligation product (R_{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.*

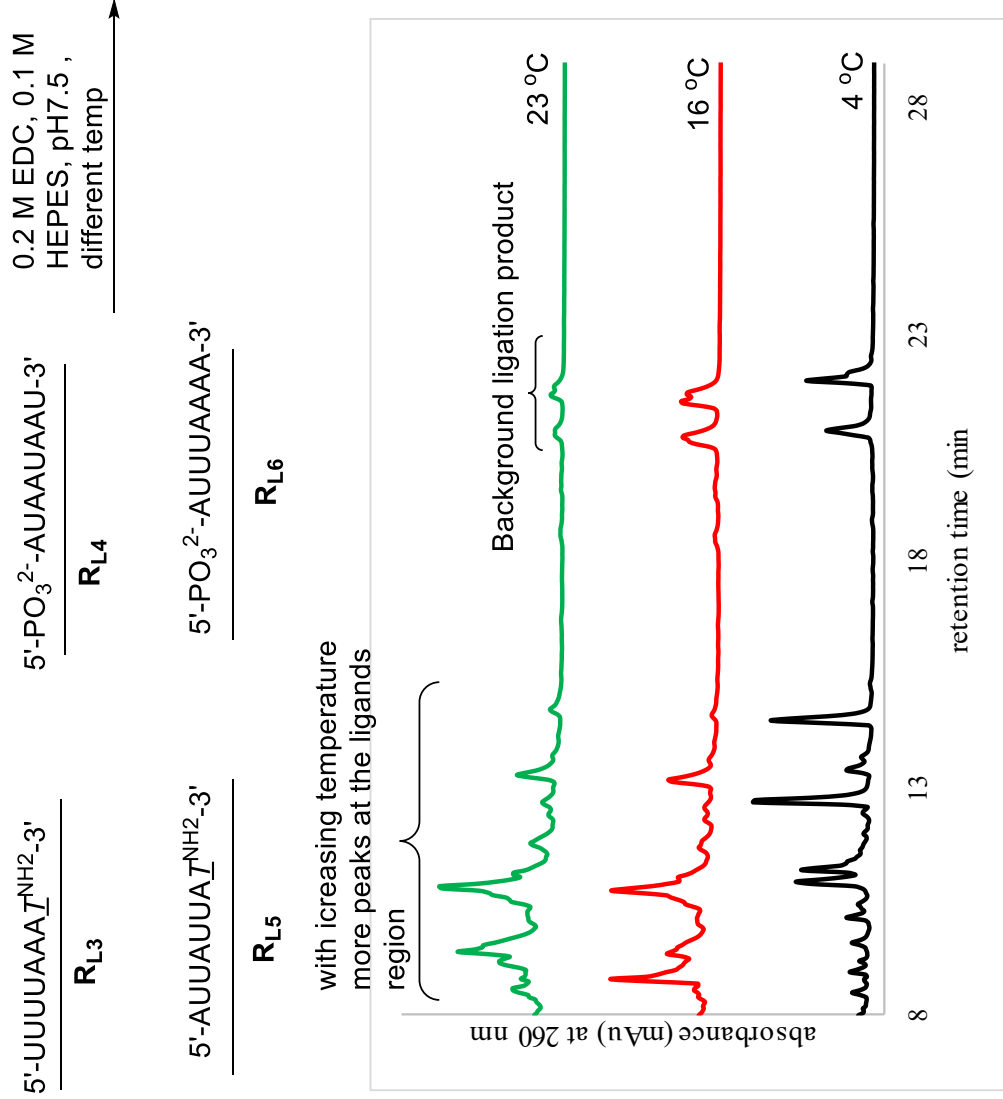
Control ligation reaction. 0.2 mM all four RNA ligands and no template



Entry	Sequence
Ligand R _{L3}	5'-UUUUAAA <u>T</u> ^{NH2} -3'
Ligand R _{L4}	5'-PO ₃ ²⁻ -AUAAUAAU-3'
Ligand R _{L6}	5'-PO ₃ ²⁻ -AUUUUAAAA-3'
Ligand R _{L5}	5'-AUUAUUA <u>T</u> ^{NH2} -3'
Background reaction	24 h
product	Peak-1 Peak-2 Peak-3
Yield(%) w.r.t. 0.1 mM external standard dT ₂₄	33% 20% 13%

Supplementary Figure 70. The AEC traces of the one-pot reaction 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 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.*

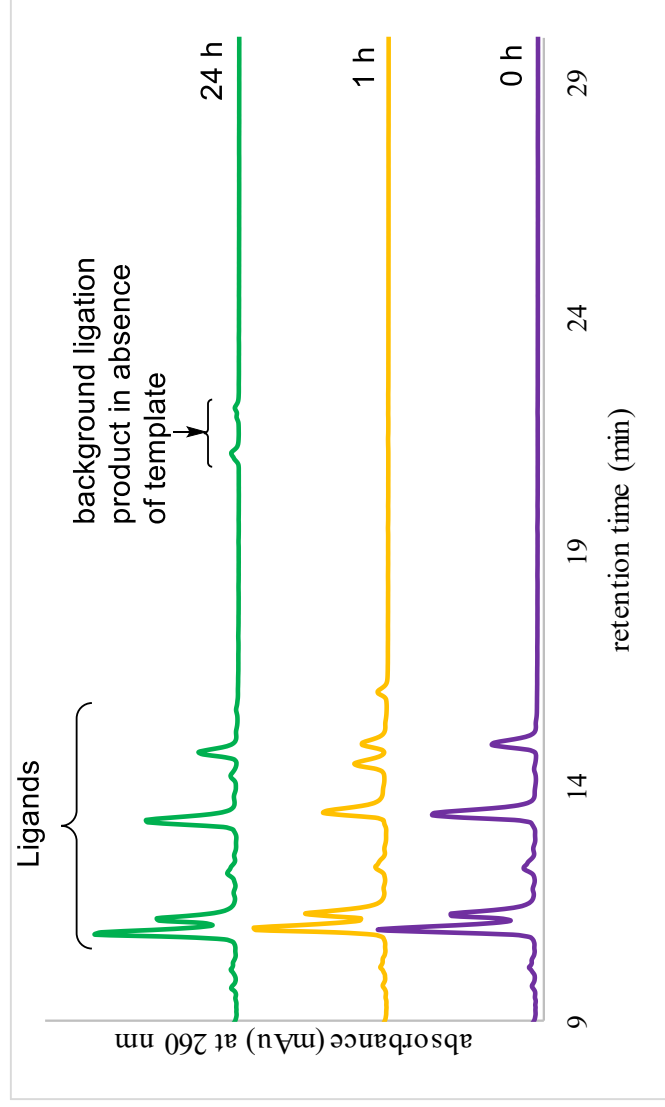
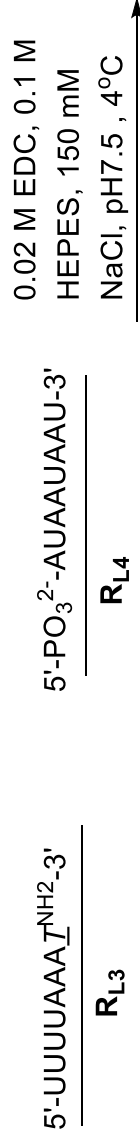
Temperature effect in background ligation reaction.



Entry	Sequence
Ligand R_{L3}	$\text{5'-UUUUAAA}\underline{\text{T}}^{\text{NH}_2}\text{-3'}$
Ligand R_{L4}	$\text{5'-PO}_3^{2-}\text{-AUAAUAAU-3'}$
Ligand R_{L6}	$\text{5'-PO}_3^{2-}\text{-AUUUUAAAA-3'}$
Ligand R_{L5}	$\text{5'-AUUAUUAA}\underline{\text{T}}^{\text{NH}_2}\text{-3'}$

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.*

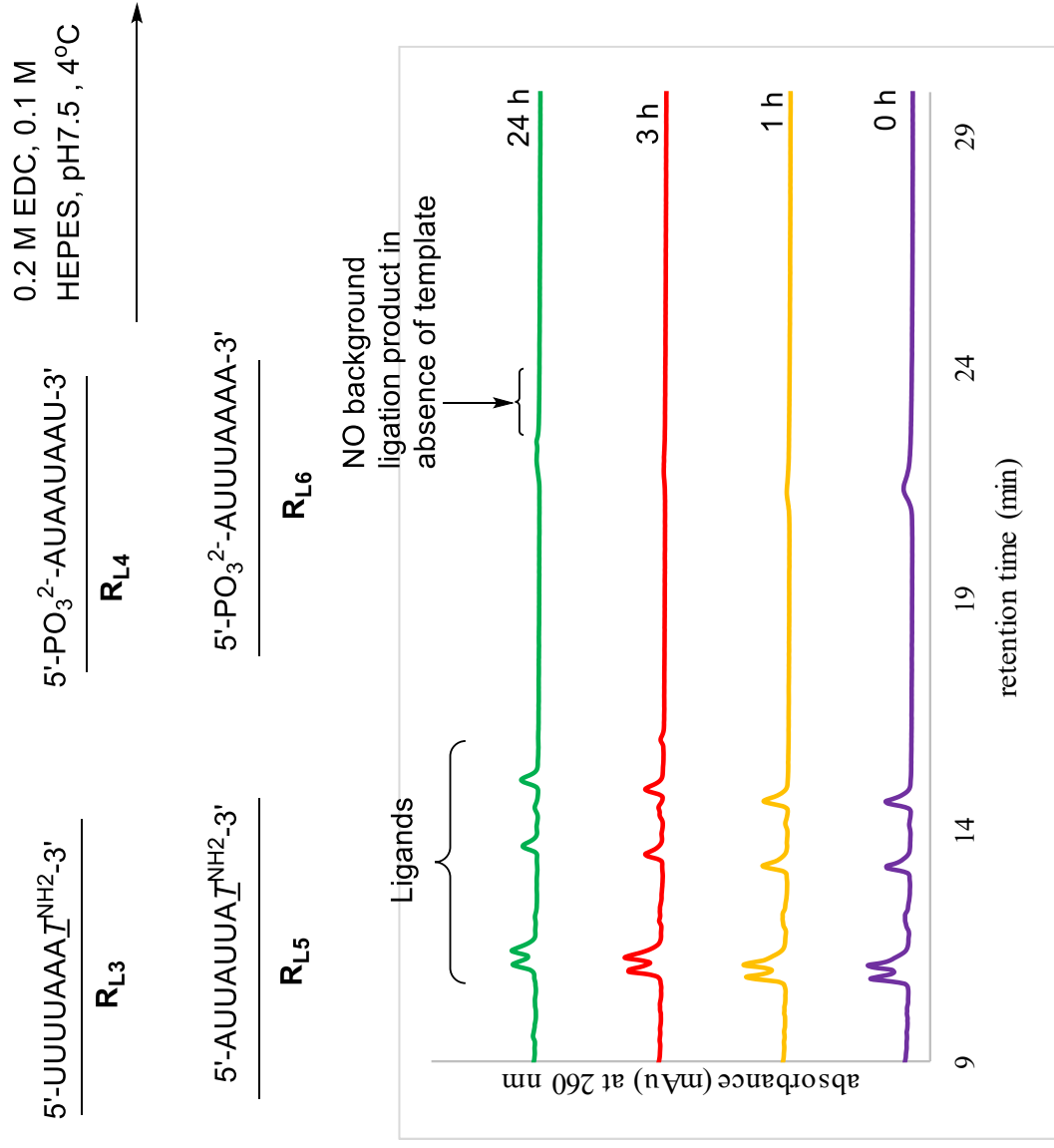
Testing EDC effect in background ligation reaction.



Entry	Sequence
Ligand R_{L3}	$5'\text{-UUUUAAA}\underline{\text{T}}^{\text{NH}_2}\text{-3'}$
Ligand R_{L4}	$5'\text{-PO}_3^{2-}\text{-AUAAUAAU-3'}$
Ligand R_{L6}	$5'\text{-PO}_3^{2-}\text{-AUUUUAAAA-3'}$
Ligand R_{L5}	$5'\text{-AUUAUUAA}\underline{\text{T}}^{\text{NH}_2}\text{-3'}$

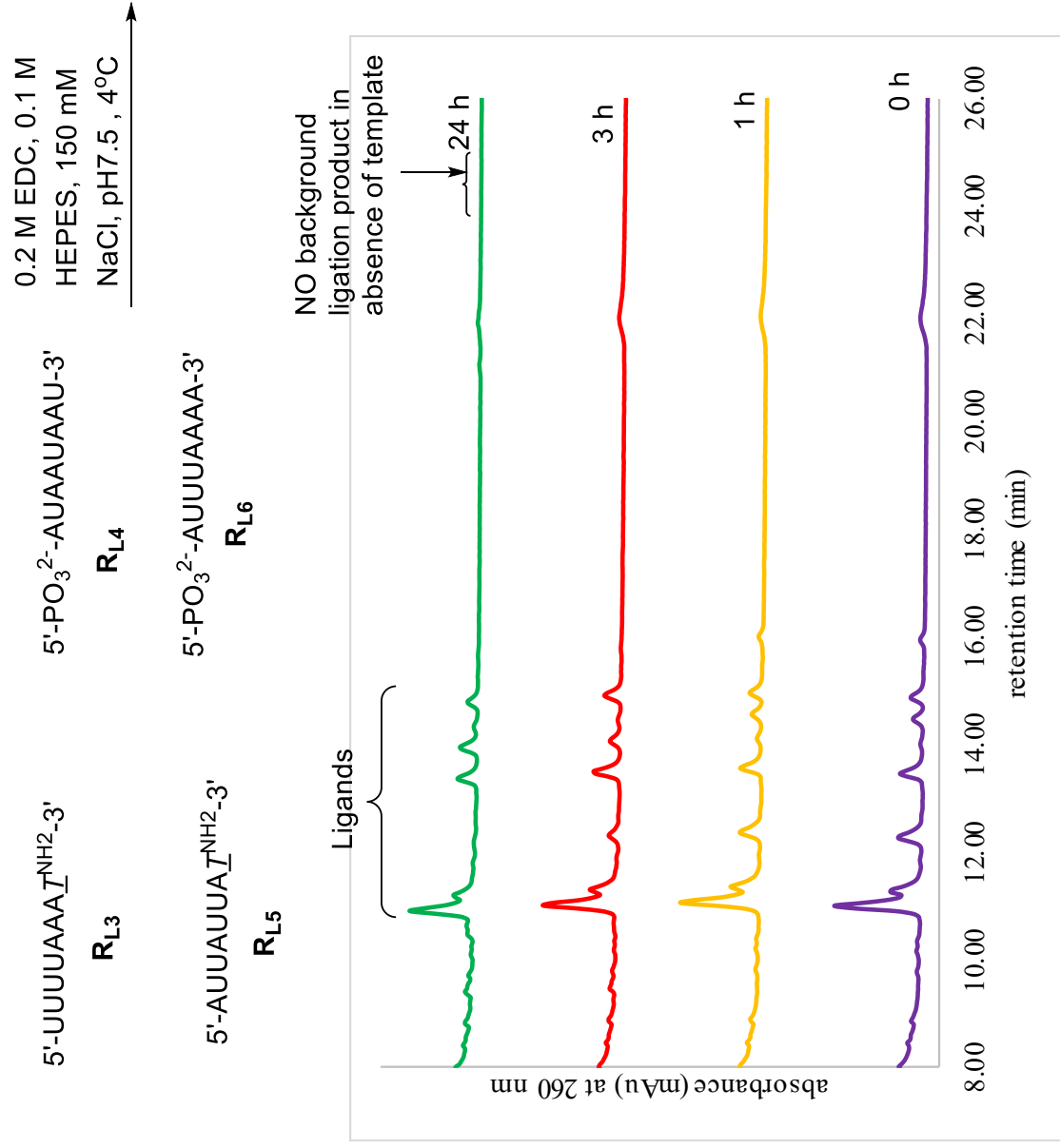
Supplementary Figure 72. The AEC traces of the 24 h one-pot reaction mixture containing $\text{R}_{\text{L3}}, \text{R}_{\text{L4}}, \text{R}_{\text{L5}}, \text{R}_{\text{L6}}$ (0.2 mM of each) in absence of any template in 0.02M EDC in 0.1M HEPES, 150 mM NaCl pH 7.5 buffer. The AEC traces indicates the appearance of new 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.



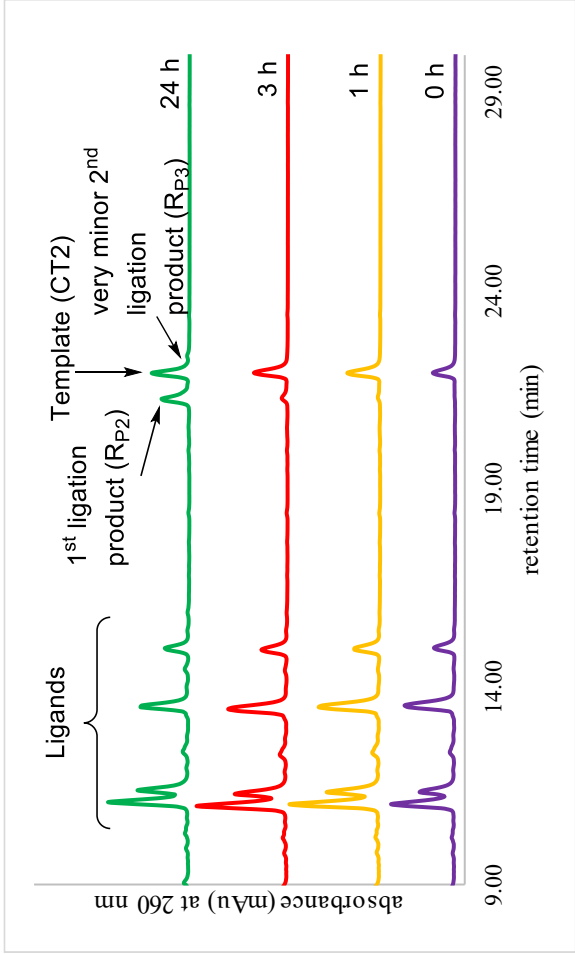
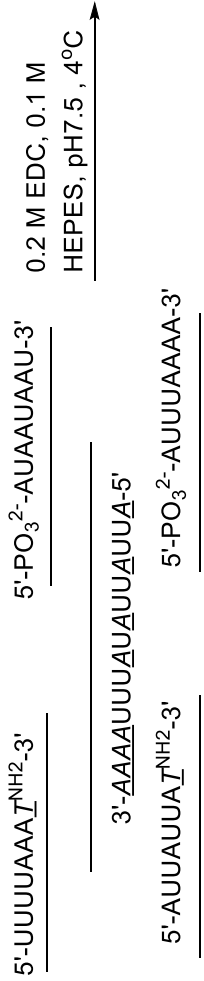
Supplementary Figure 73. The AEC traces of the 24 h one-pot reaction mixture containing $\text{R}_{\text{L3}}, \text{R}_{\text{L4}}, \text{R}_{\text{L5}}, \text{R}_{\text{L6}}$ (20 μM of each) in absence of any template in 0.2M EDC in 0.1M HEPES, pH 7.5 buffer. The AEC traces indicates that no background 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 μ M all four RNA ligands and no template



Supplementary Figure 74. The AEC traces of the 24 h one-pot reaction mixture containing $\mathbf{R_{L3}}$, $\mathbf{R_{L4}}$, $\mathbf{R_{L5}}$, $\mathbf{R_{L6}}$ (20 μ M 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.*

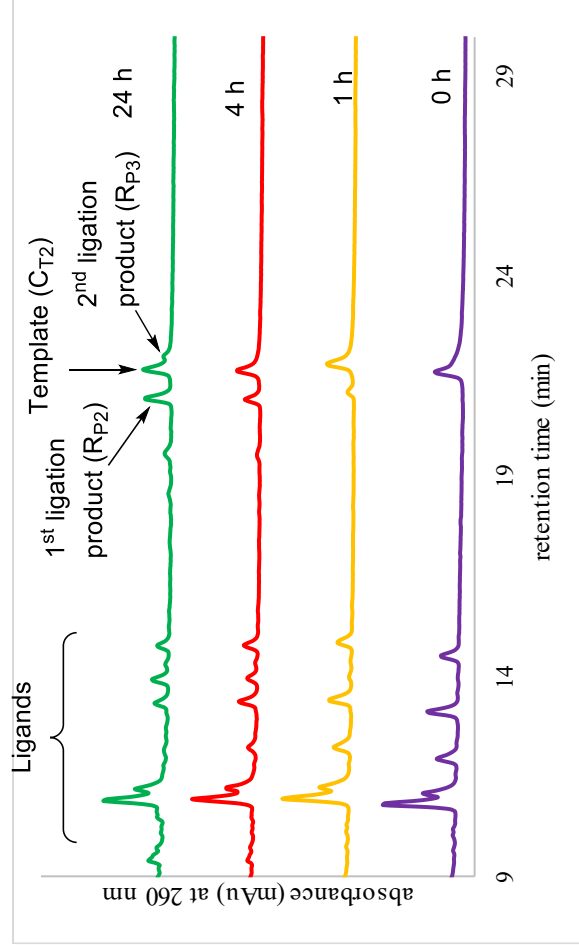
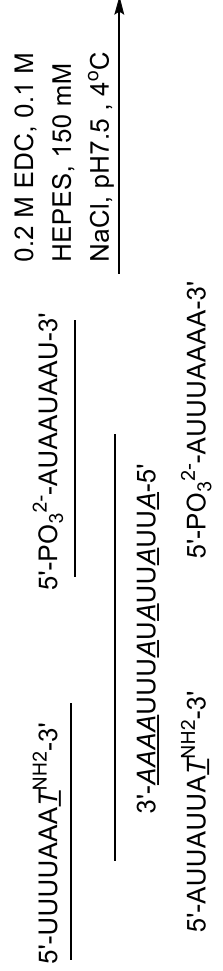
One-pot replication cycle. All four RNA ligands (20 μ M each) on RDNA chimeric template (10 μ M)



Entry	Sequence
Ligand R _{L3}	5'-UUUUAAA <u>T</u> ^{NH2} -3'
Ligand R _{L4}	5'-PO ₃ ²⁻ -AUAAUAAU-3'
Ligand R _{L6}	5'-PO ₃ ²⁻ -AUUUUAAAA-3'
Ligand R _{L5}	5'-AUUAUU <u>A</u> ^{NH2} -3'
Template C _{T2}	3'-AAAAUUU <u>AU</u> AUU <u>AU</u> UA-5'
1 st Ligation	5'-AUUUAAAA(NH) <u>T</u> AUU <u>AU</u> UA-3'
product R _{P2}	1 h 3 h 24 h
Yield (%)	<5% 12% 69%
2 nd Ligation	3'-AAAAUUU <u>A</u> (NH) <u>T</u> AUU <u>AU</u> UA-5'
product R _{P3}	1 h 3 h 24 h
Yield (%)	0% 0% <5%

Supplementary Figure 75. The AEC traces of the one-pot reaction mixture containing R_{L3},R_{L4}, R_{L5},R_{L6} (20 μ M of each) on DNA-RNA chimeric C_{T2} template (10 μ M) in 0.2MEDC 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.*

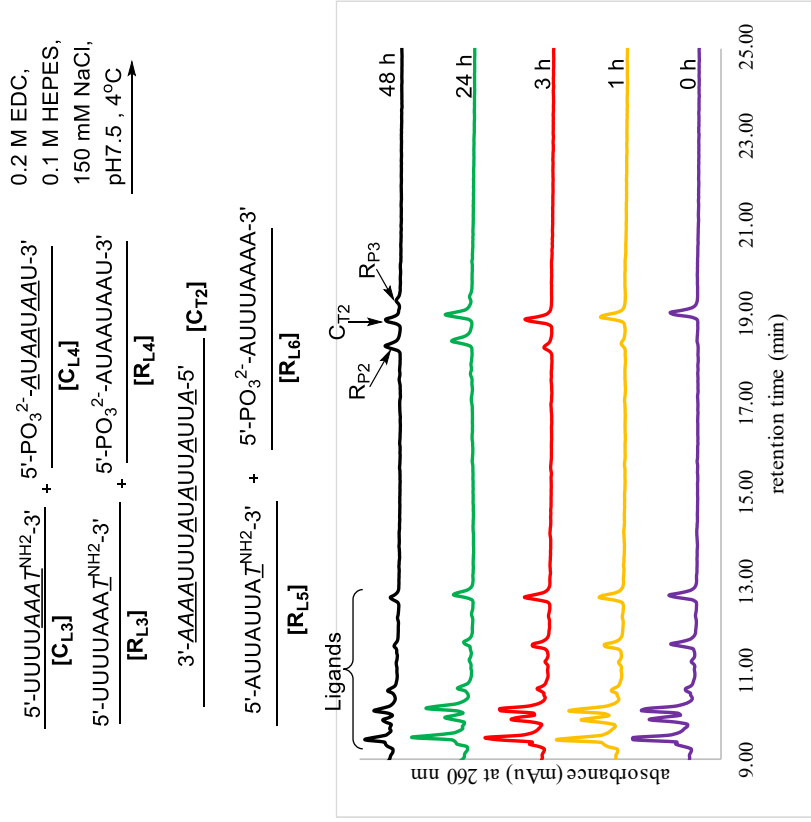
One-pot replication cycle. All four RNA ligands (20 μ M each) on RDNA chimeric template (10 μ M)



Entry	Sequence
Ligand R _{L3}	5'-UUUUAAA <u>T</u> ^{NH2} -3'
Ligand R _{L4}	5'-PO ₃ ²⁻ -AUAAUAAU-3'
Ligand R _{L6}	5'-PO ₃ ²⁻ -AUUUUAAAA-3'
Ligand R _{L5}	5'-AUUAAUUA <u>T</u> ^{NH2} -3'
Template C _{T2}	3'- <u>AAAAUUUUAAU</u> <u>AUU</u> <u>AUU</u> A-5'
1 st Ligation product R _{P2}	5'-AUUUAAAA(NH) <u>T</u> AUUAAUUA-3'
Yield (%)	1 h 4 h 24 h
	14% 45% 83%
2 nd Ligation product R _{P3}	3'-AAAAUUUA(NH) <u>T</u> AUUAAUUA-5'
Yield (%)	1 h 4 h 24 h
	0% 0% 18%

Supplementary Figure 76. The AEC traces of the one-pot reaction mixture containing R_{L3}, R_{L4}, R_{L5}, R_{L6} (20 μ M of each) on DNA-RNA chimeric C_{T2} template (10 μ M) in 0.2M EDC in 0.1M HEPES, 150 mM NaCl, pH 7.5 buffer. The AEC traces indicates both the 1st ligation product and 2nd 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.

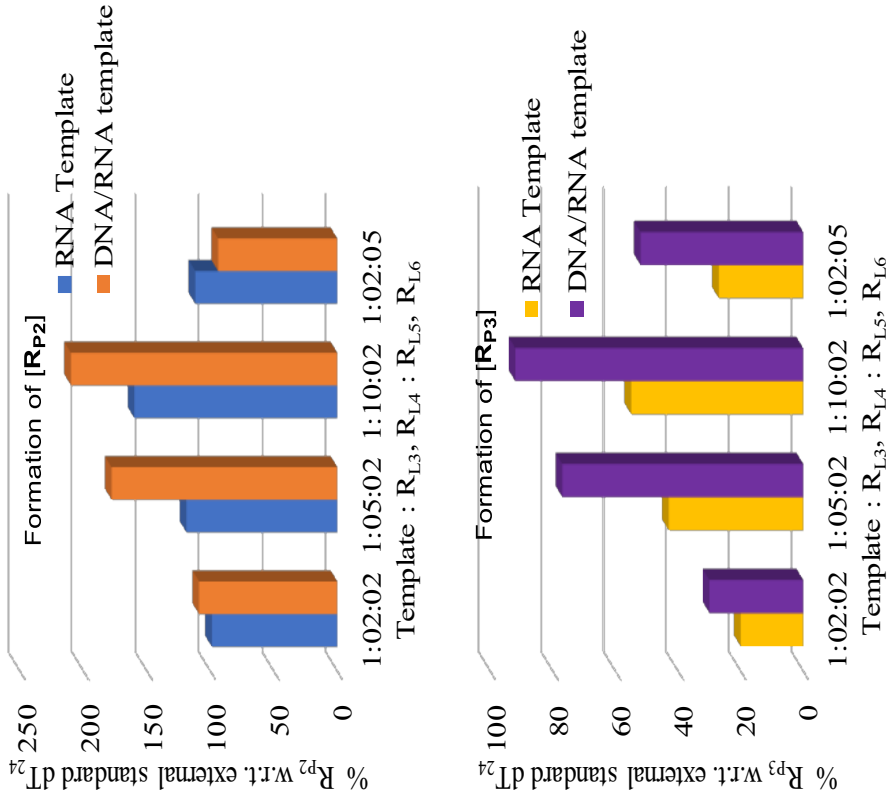
One-pot replication cycle. All RNA (R_{L3} , R_{L4} , R_{L5} , R_{L6}) ligands and RDNA chimeric (C_{L3} , C_{L4}) ligands on RDNA chimeric template.



Entry	Sequence
Ligand R_{L3}	5'-UUUUUUAAA <u>T</u> ^{NH₂-3'}
Ligand R_{L4}	5'-PO ₃ ²⁻ -AUAAUAAU-3'
Ligand C_{L3}	5'-UUUUUUAAA <u>T</u> ^{NH₂-3'}
Ligand C_{L4}	5'-PO ₃ ²⁻ -AUAAUAAU-3'
Ligand R_{L6}	5'-PO ₃ ²⁻ -AUUUAAAAA-3'
Ligand R_{L5}	5'-AUUAUUUA <u>T</u> ^{NH₂-3'}
Template C_{T2}	3'- <u>AAAAUUUAUAAUAAUUA</u> -5'
1 st Ligation	5'-UUUUUUAAA <u>T</u> (NH)AUAAUAAU-3'
product R_{P2}	24 h
Yield (%)	83%
2 nd Ligation	3'-AAAAUUUA(NH) <u>TAUUAAUUA</u> -3'
product R_{P3}	24 h
Yield (%)	16%
	31%

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

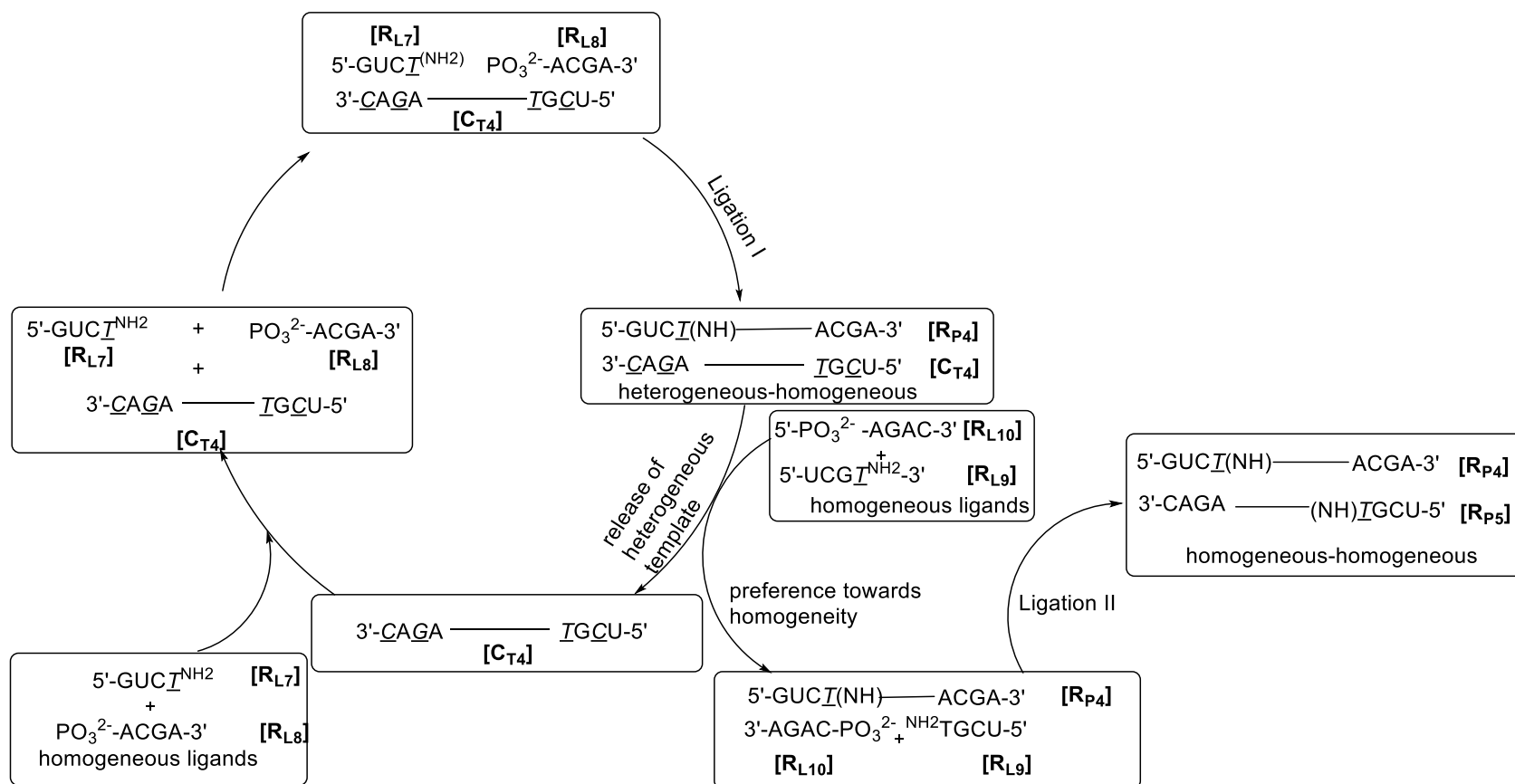
Variable ligands effect. Comparison of the formation of R_{P2} and R_{P3} on RNA R_{T2} and chimeric RDNA C_{T2} template in 48 h of one-pot self-replication cycle with 10 μ M template and varied ligands concentrations.



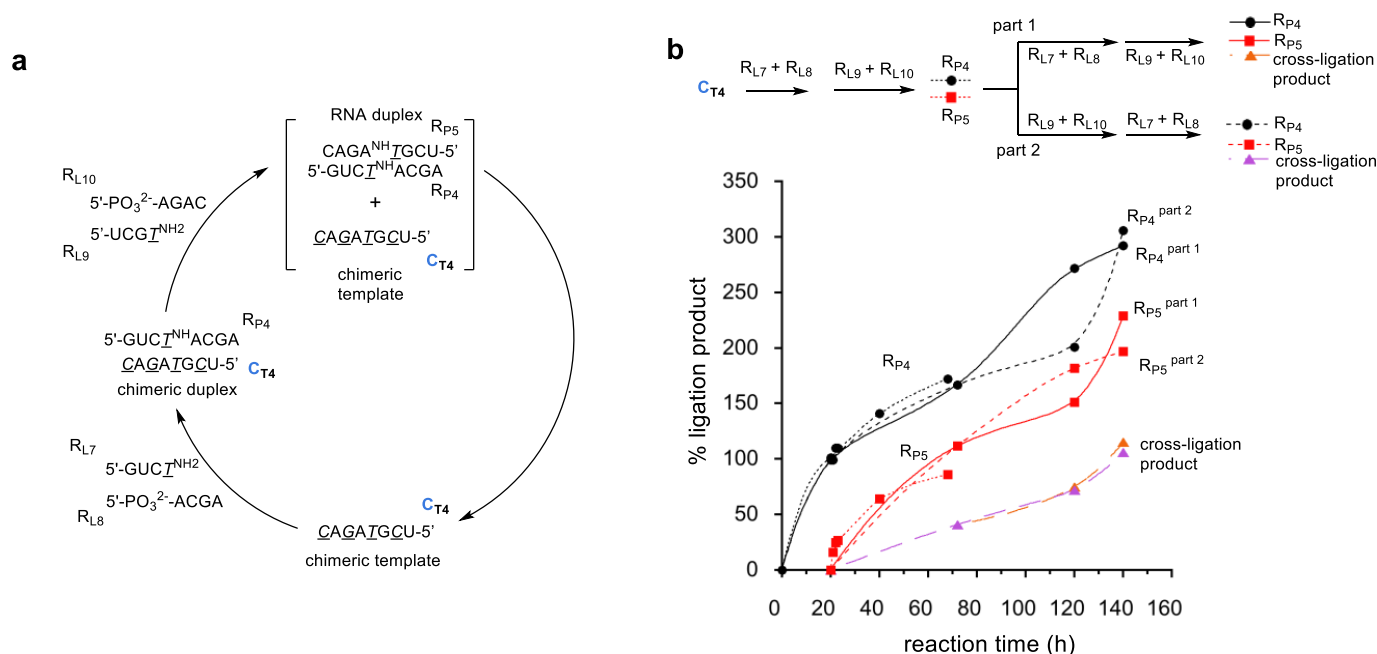
Ligation product	Template: R_{L3} , R_{L4} : R_{L5} , R_{L6}	[R_{T2}] RNA Template	[C_{T2}] DNA-RNA Chimeric Template
1 st Ligation product R_{P2}	1:2:2	99%	109%
% Yield	1:5:2	119%	178%
	1:10:2	160%	210%
2 nd Ligation product R_{P3}	1:2:5	112%	94%
% Yield	1:2:2	18%	30%
	1:5:2	43%	77%
	1:10:2	55%	92%
	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 μ M template concentration in 0.2 MEDC, 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 $\pm$ 5%.

Design of short (A,U/T,G,C) RDNA chimeric sequence-based replicating system:

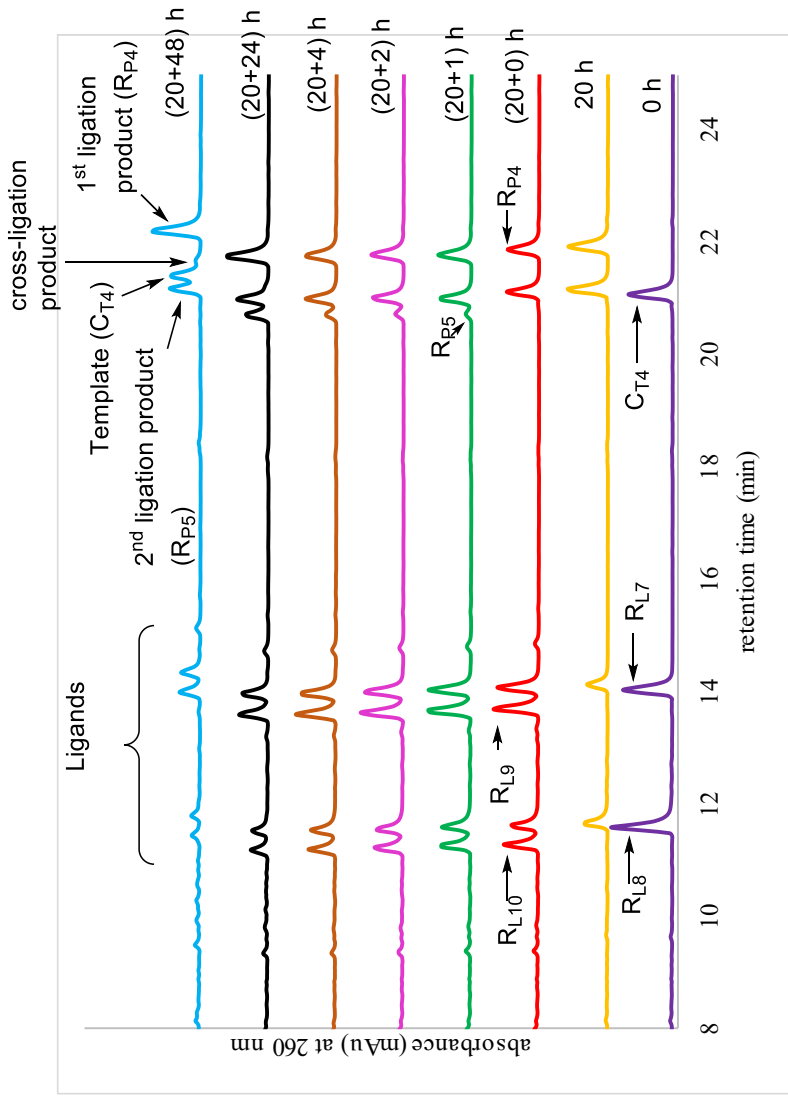


Supplementary Figure 79. All four-nucleobase containing octameric DNA-RNA chimeric system based replicating cycle. It is envisaged that the addition of ligands (R_{L7}, R_{L8}), complementary to the 1st ligation product (R_{P4}) will release the chimeric template (C_{T4}) due to the preference of homogeneous 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 (AUGC)-RDNA template C_{T4} with RNA ligands R_{L7}, R_{L8} produces R_{P4}, which in the presence of R_{L9}, R_{L10} forms R_{P5} and releases the C_{T4} 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 $\pm 5\%$. The stepwise addition of ligands (R_{L7}+R_{L8}, followed by R_{L9}+R_{L10}) to the template (C_{T4}) at 4°C led to the formation of the first ligation product (R_{P4}) followed by the second ligation (R_{P5}) product. Moreover, the formation and presence of the second ligation product (R_{P5}) in the above mixture resulted in an increase in the amount of the first ligation product (R_{P4}) suggesting a positive feedback for each other's production, again affirming the ability of the chimeric-template C_{T4} 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 R_{L8}+R_{L9} and R_{L7}+R_{L10}. 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 C_{T4}, 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 μ M 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 R_{L7}+R_{L8} and after 48 h followed by R_{L9}+R_{L10}, whereas to part 2 we reversed the sequence of addition (R_{L9}+R_{L10} followed, at 48 h, by R_{L7}+R_{L8}) and monitored the progress of the ligation reactions (Supplementary Figs. 83-84). In both cases, the continuous and sequential increase of respective products R_{P4} and R_{P5} was observed (Fig. 80b) and was accompanied by formation of cross-ligation products (R_{L7}+R_{L10} and R_{L9}+R_{L8}, Supplementary Figs. 85-86).

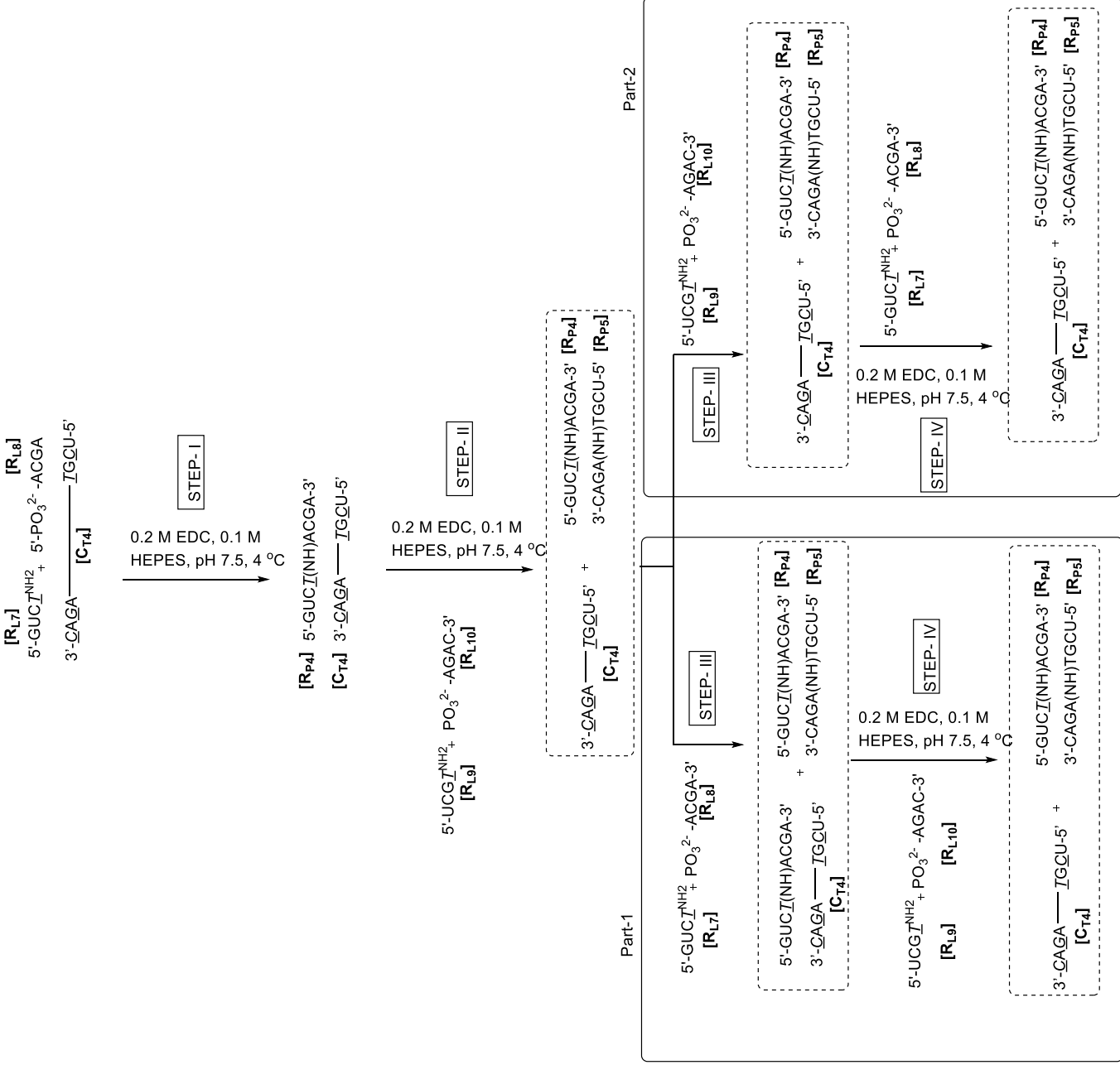
Short sequence replication cycle. Development of short (A,U/T,G,C) RDNA chimeric template-based replication cycle.



Entry	Sequence present	% R _{P4} formed	% R _{P5} formed	% cross-ligation product
0 h	C _{T4} , R _{L7} , R _{L8}	0	-	-
20 h	C _{T4} , R _{P4} , R _{L7} , R _{L8}	99	-	-
(20+0) h	C _{T4} , R _{P4} , R _{L7} , R _{L8} , R _{L9} , R _{L10}	99	0	0
(20+1) h	C _{T4} , R _{P4} , R _{P5} , R _{L7} , R _{L8} , R _{L9} , R _{L10}	99	16	0
(20+2) h	C _{T4} , R _{P4} , R _{P5} , R _{L7} , R _{L8} , R _{L9} , R _{L10}	110	25	0
(20+3) h	C _{T4} , R _{P4} , R _{P5} , R _{L7} , R _{L8} , R _{L9} , R _{L10}	110	27	0
(20+20) h	C _{T4} , R _{P4} , R _{P5} , R _{L7} , R _{L8} , R _{L9} , R _{L10}	141	64	0
(20+48) h	C _{T4} , R _{P4} , R _{P5} , R _{L7} , R _{L8} , R _{L9} , R _{L10}	172	86	16

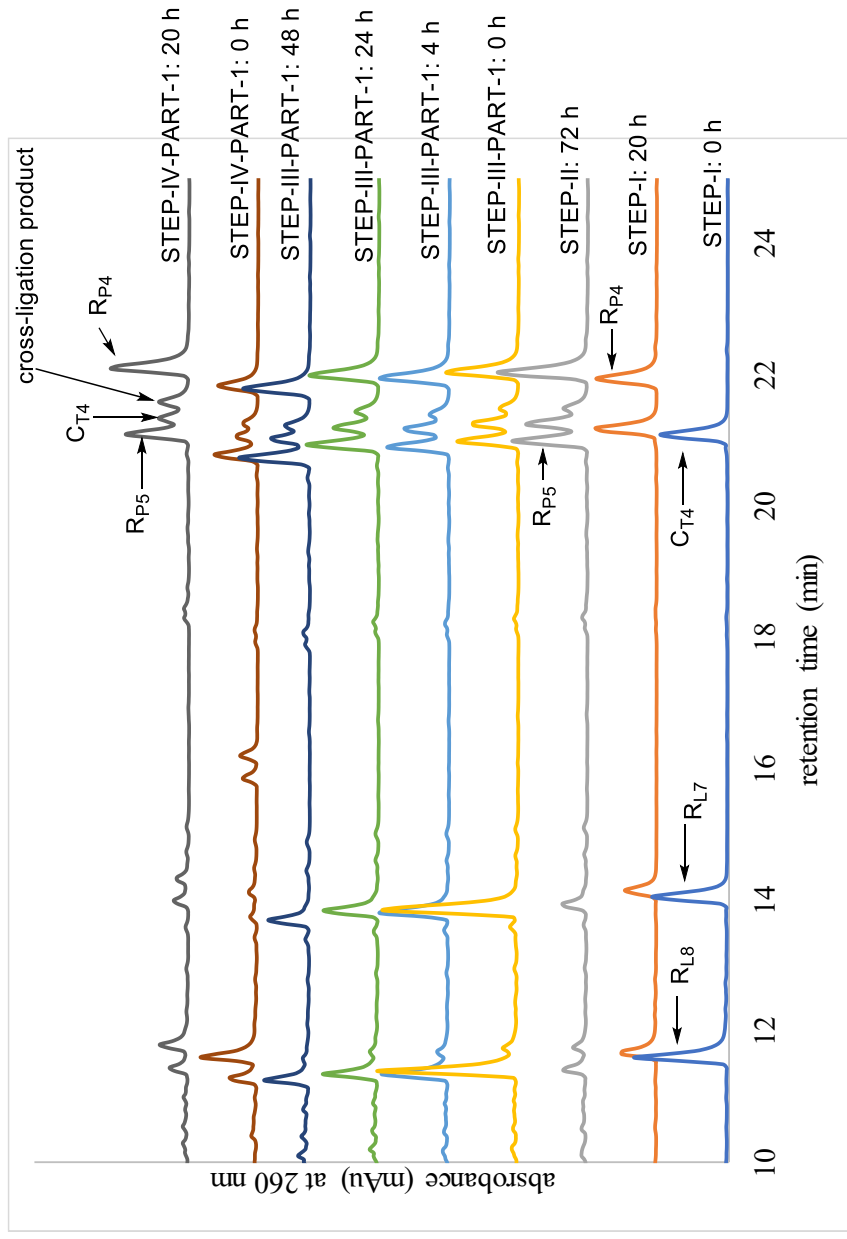
Supplementary Figure 81: The AEC traces of the heterogeneous DNA-RNA template mediated emergence of homogeneous sequences. Similar to the hexadecameric A,U template (C_{T7}), the template as short as octameric (C_{T4}), also able to complete the replicating cycle. Bottom two traces (0 h and 20 h) indicates the emergence of 1st ligation product (R_{P4}) in presence of homogeneous ligands (R_{L7}, R_{L8}) on heterogeneous template (C_{T4}, 0.1 mM). After 20 h of reaction two homogeneous ligands (R_{L9}, R_{L10}) complementary to R_{P4} was added and the reaction was monitored up to 48 h and the AEC traces indicates the appearance of 2nd ligation product (R_{P5}) beside initial template (C_{T4}) and 1st ligation product (R_{P4}) (top five spectra). During that reaction the final ratio of C_{T4}: R_{L7}, R_{L8}: R_{L9}, R_{L10} was (1:2:2). Gradient: 0 to 20% Buffer B in 1 min then 20 to 90% Buffer B in 30 min.

Continuity of replication cycle. Testing the dynamic nature of the short sequence-based replication cycle.



Supplementary Figure 82: Schematic representation of the dynamic nature of the DNA-RNA octameric heterogeneous template based replicating cycle where the ligands R_{L7}, R_{L8} were continuously feeding to check the dynamic nature of the cycle. The reaction was started with R_{L7}, R_{L8} (0.2 mM each) and template (C_{T4} , 0.1 mM). After 20 h, it shows the formation of 1st ligation product (R_{P4}) and to that 20 h old reaction mixture ligands R_{L9}, R_{L10} (complementary to 1st ligation product (R_{P4}) were added and the reaction was allowed for another 72 h and then divided into two parts- in one part R_{L7}, R_{L8} and in another part R_{L9}, R_{L10} 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 R_{L7}, R_{L10} and R_{L8}, R_{L9} were also observed.

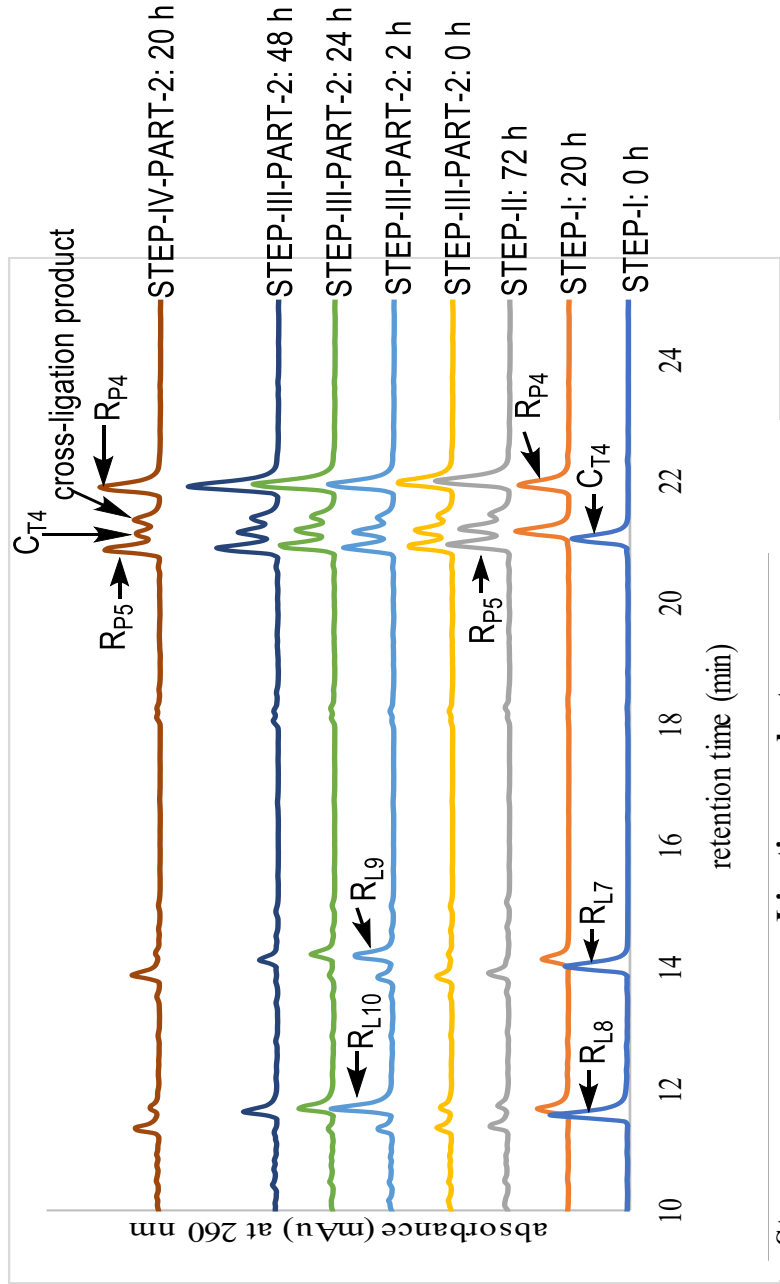
Continuity of part-1 of the replication cycle on RDNA template



Step	Ligation product		
Step I	R_{P4}		
Time	20 h		
%Yield	101%		
Step II	R_{P4}	R_{P5}	Cross
Time	72 h		
%Yield	167%	112%	41%
Step III, Part-I	R_{P4}	R_{P5}	Cross
Time	48 h		
%Yield	272%	151%	75%
Step IV, Part-1	R_{P4}	R_{P5}	Cross
Time	20 h		
%Yield	292%	229%	115%

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.

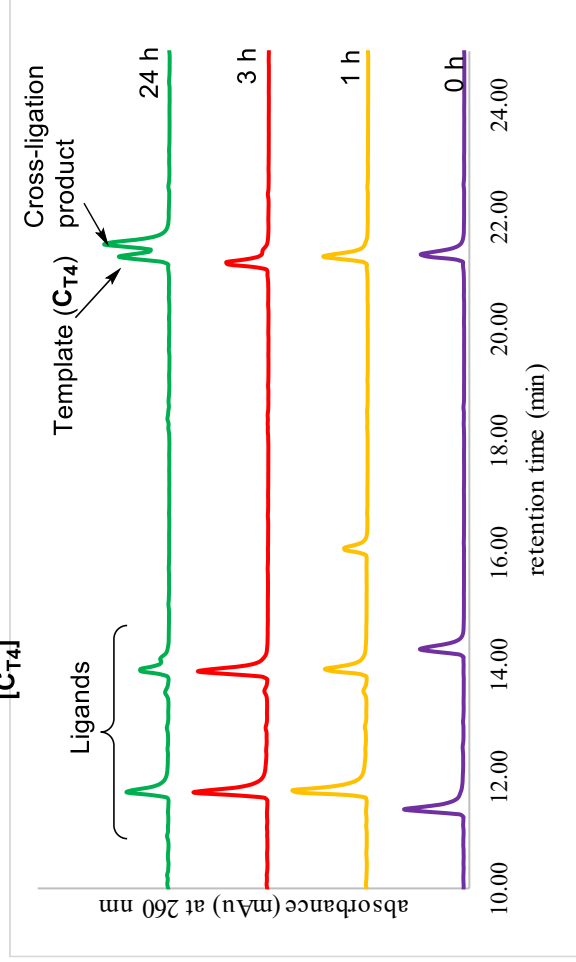
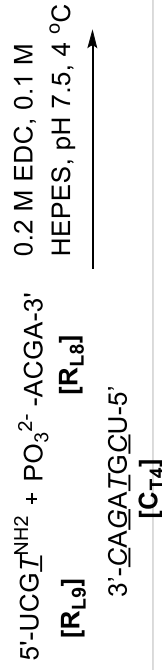
Continuity of part-2 of the replication cycle on RDNA template



Step	Ligation product		
Step I	R _{P4}		
Time	20 h		
%Yield	101%		
Step II	R _{P4}	R _{P5}	Cross
Time	72 h		
%Yield	167%	112%	41%
Step III, Part-2	R _{P4}	R _{P5}	Cross
Time	48 h		
%Yield	201%	182%	72%
Step IV, Part-2	R _{P4}	R _{P5}	Cross
Time	20 h		
%Yield	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.*

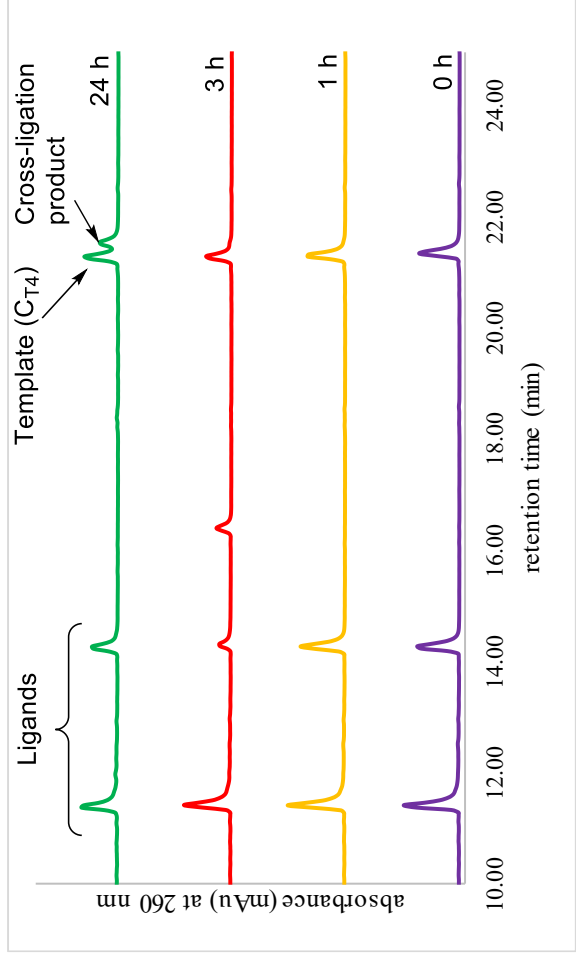
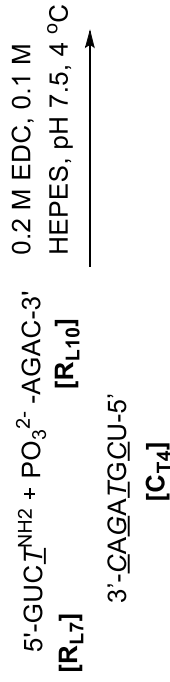
Cross Ligation reaction: RNA (R_{L8}, R_{L9}) ligands on chimeric RDNA C_{T4} template



Entry	Sequence	Expected mass	Observed mass
Ligand R_{L9}	5'-UCG $\underline{T}^{\text{NH}_2}$	1199	1199
Ligand R_{L8}	5'-PO $_3^{2-}$ -ACGA-3'	1326	1328
Template C_{T4}	3'- $\underline{\text{CAGA}}\underline{\text{TGCU}}$ -5'	2459	2461
Cross-Ligation product	5'-UCG $\underline{T}(\text{NH})$ ACGA-3'	2506	2508
Yield (%)	24 h		
	134%		

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_{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.*

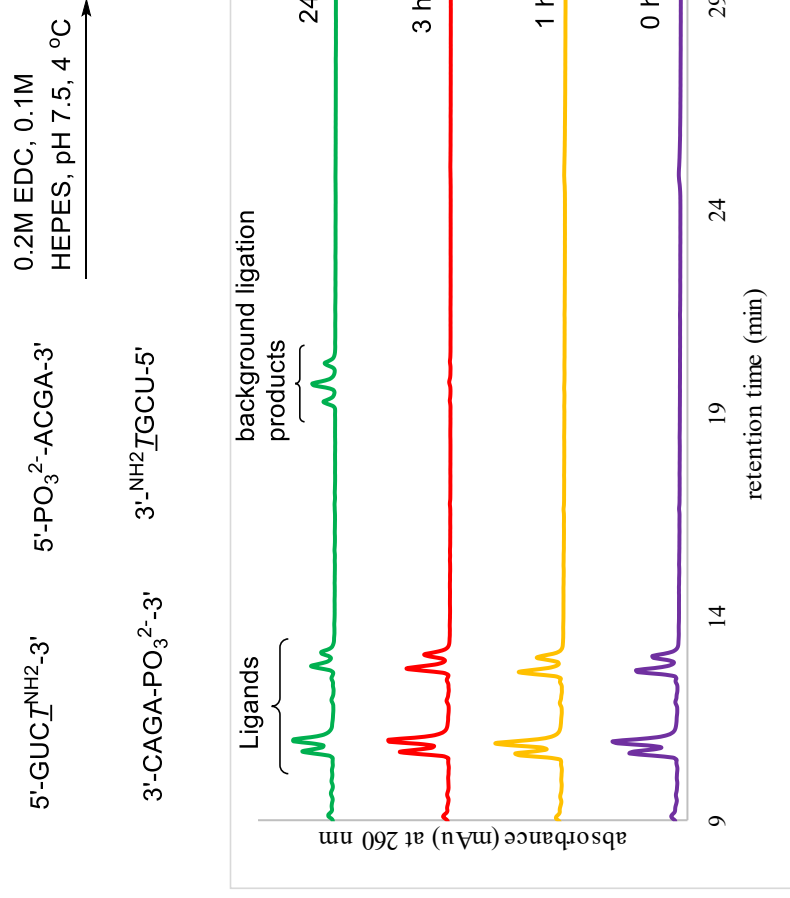
Cross Ligation reaction: RNA (R_{L7}, R_{L9}) ligands on chimeric RDNA C_{T4} template



Entry	Sequence	Expected mass	Observed mass
Ligand R_{L7}	5'-GUC $\underline{T}^{\text{NH}_2}$	1199	1198
Ligand R_{L10}	5'-PO $_3^{2-}$ -AGAC-3'	1326	1327
Template C_{T4}	3'- $\underline{\text{CAGA}}\underline{\text{TGC}}\underline{\text{U-5'}}$	2459	2460
Ligation product	5'-GUC $\underline{T}(\text{NH})$ AGAC-3'	2506	2506
Yield (%)	24 h		
	64%		

Supplementary Figure 86: The AEC traces of the cross-ligation reaction between homogeneous RNA (R_{L7}, R_{L10}) 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.*

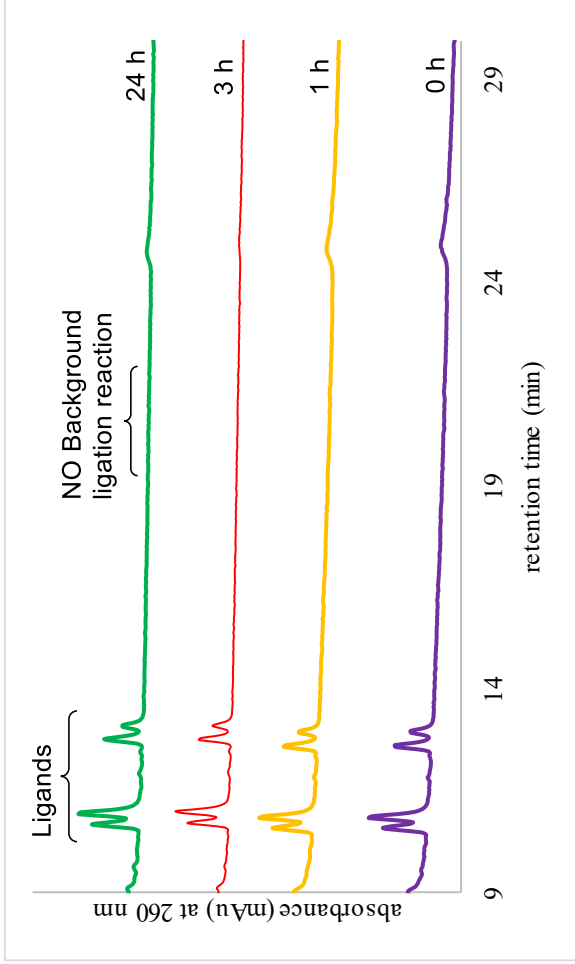
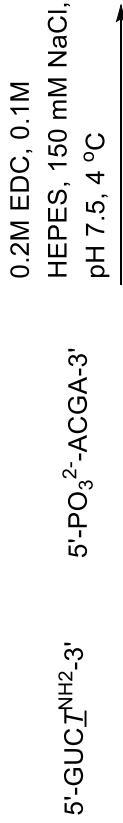
Background ligation reaction in short sequence.



Entry	Sequence	Expected mass	Observed mass
Ligand R_{L7}	$5'\text{-GUC}\overline{\text{T}}^{\text{NH}_2}$	1199	1198
Ligand R_{L8}	$5'\text{-PO}_3^{2-}\text{-ACGA-}3'$	1326	1327
Ligand R_{L10}	$3'\text{-CAGA-PO}_3^{2-}\text{-}5'$	1326	1327
Ligand R_{L9}	$3'\text{-NH}_2\overline{\text{T}}\text{GCU-}5'$	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_{L7} , R_{L8} , R_{L9} , R_{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.*

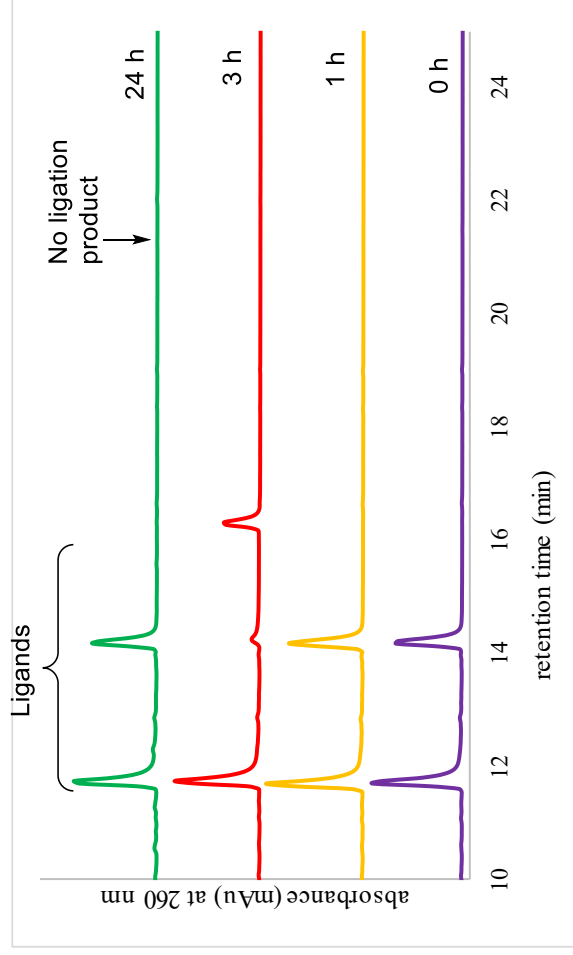
Background ligation reaction in short sequence.



Entry	Sequence	Expected mass	Observed mass
Ligand R _{L7}	5'-GUC $\overline{T^{\text{NH}_2}}$	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 ₂ $\overline{\text{TGCU-}}$ 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 μ M 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.

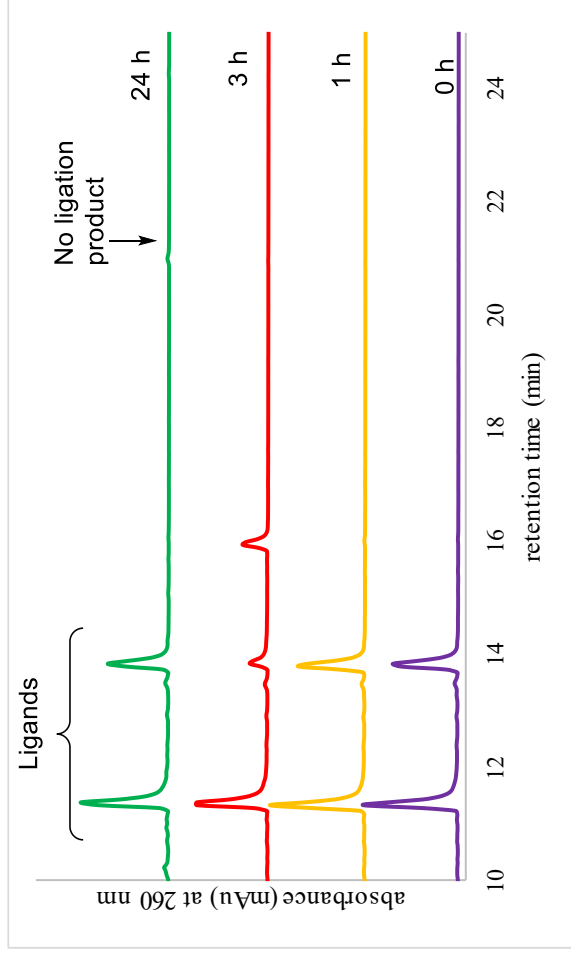
Control reaction. Homogeneous RNA ligands (R_{L7} , R_{L8}) in absence of any template



Entry	Sequence	Expected mass	Observed mass
Ligand R_{L7}	$5'\text{-GUC}\overline{I}^{\text{NH}_2}$	1199	1199
Ligand R_{L8}	$5'\text{-PO}_3^{2-}\text{-ACGA-3'}$	1326	1328
Ligation product R_{P4}	$5'\text{-GUC}\overline{I}(\text{NH})\text{ACGA-3'}$	2506	2507
Yield (%)	(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

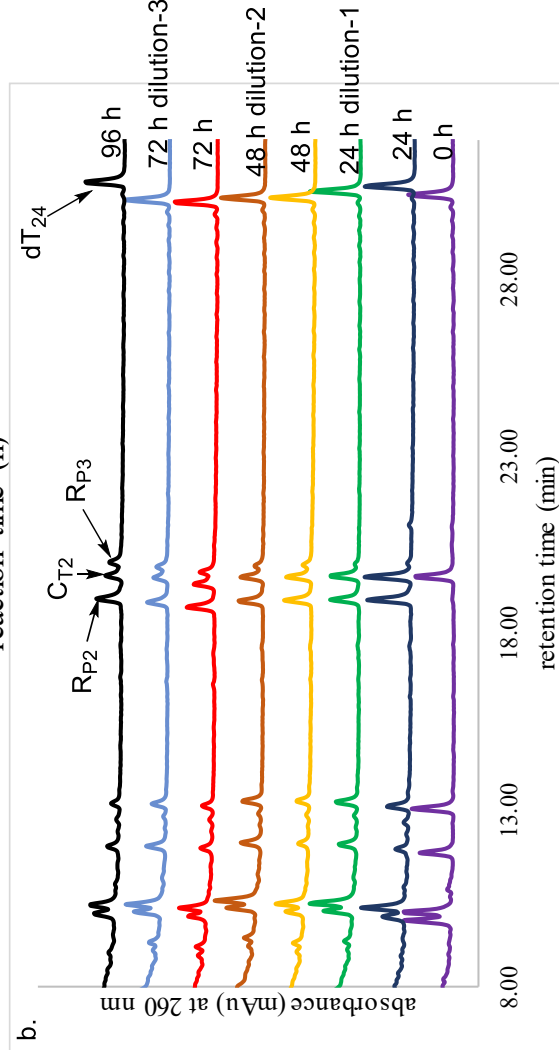
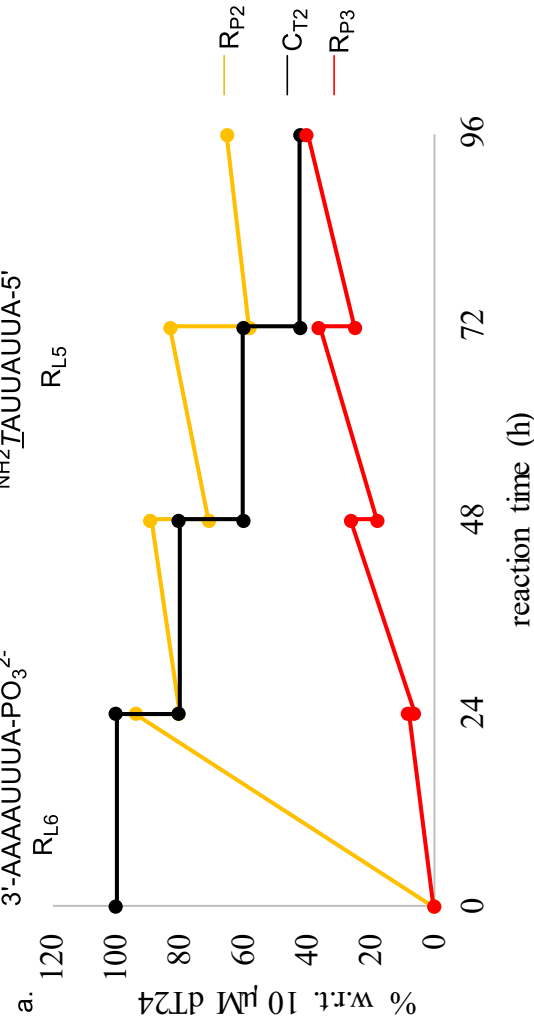


Entry	Sequence	Expected mass	Observed mass
Ligand C_{L5}	5'-GTC <u>T</u> ^{NH2}	1197	1195
Ligand C_{L6}	5'-PO ₃ ²⁻ -ACGA-3'	1294	1294
Ligation product C_{P3}	5'-GTC <u>T</u> (NH)ACGA-3'	2472	2471 (In AEC not observed)

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.*

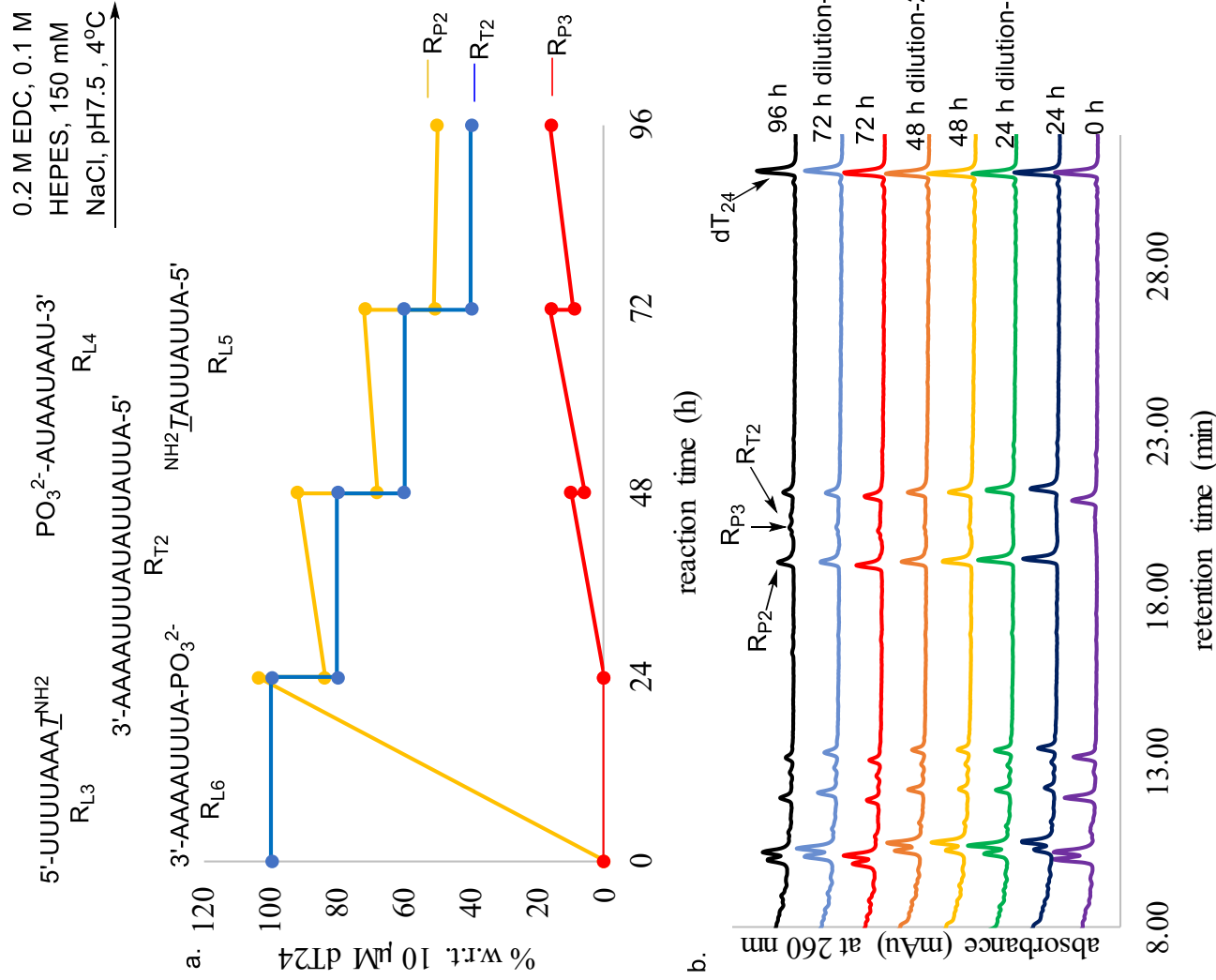
Stepwise dilution reaction. The effect of stepwise dilution on RDNA (C_{T2}) template in the presence of $R_{L3}, R_{L4}, R_{L5}, R_{L6}$ ligands:

0.2 M EDC, 0.1 M
HEPES, 150 mM
NaCl, pH7.5, 4°C



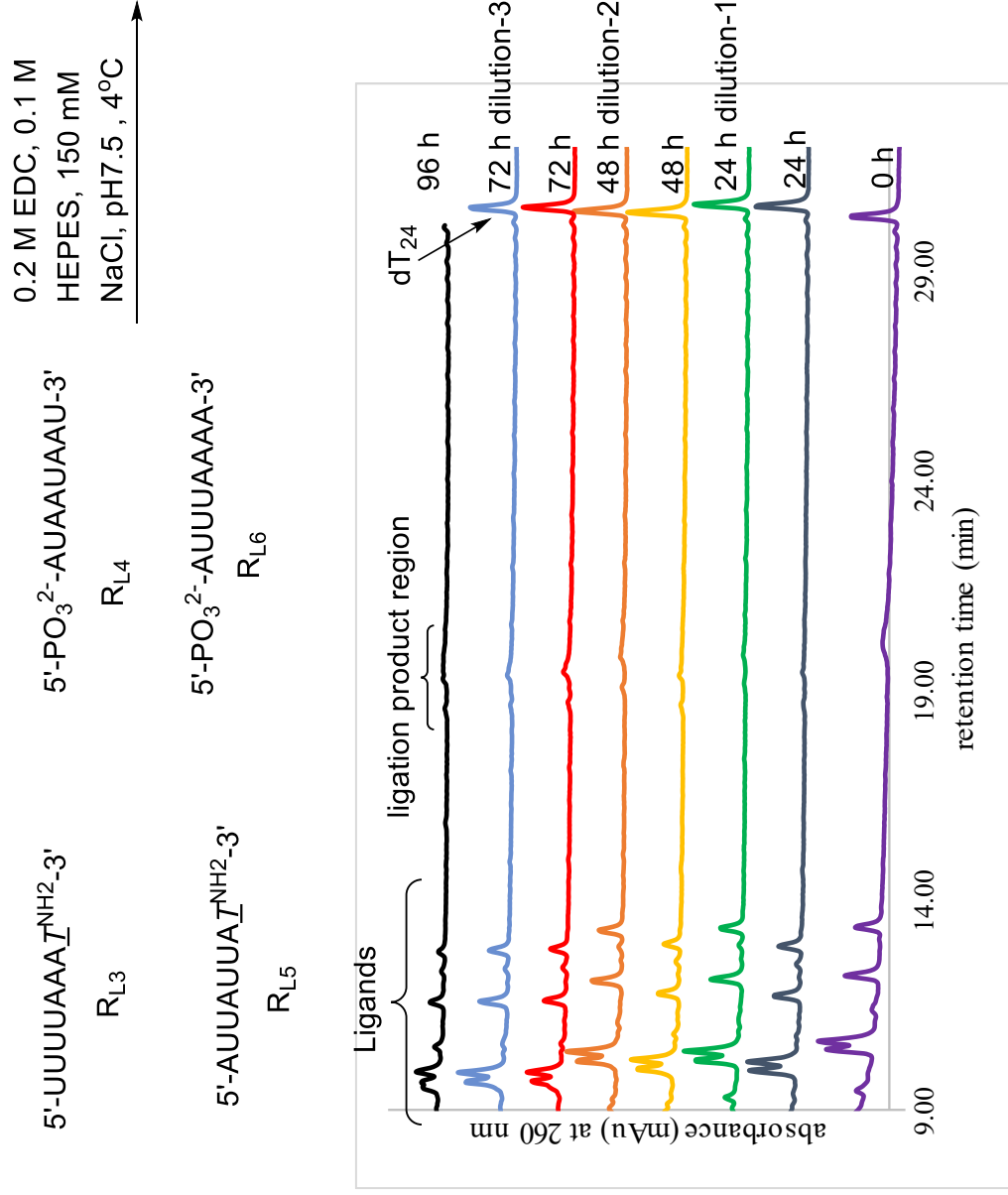
Supplementary Figure 91. The effect of stepwise dilution on RDNA chimeric C_{T2} template. The reaction was started with 10 μM template and at 24 h interval 1 μL was taken out and the reaction mixture was diluted with 1 μL of reaction buffer containing $R_{L3}, R_{L4}, R_{L5}, R_{L6}$ (20 μM of each), 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 $\pm 5\%$.

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.



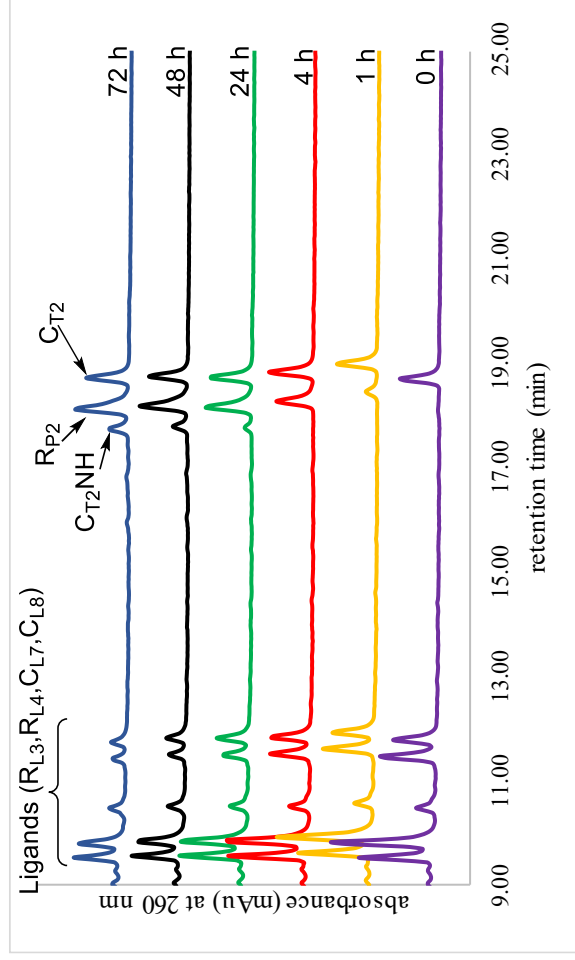
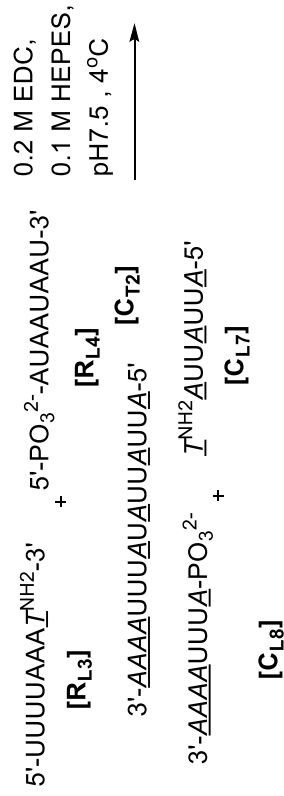
Supplementary Figure 92. The effect of stepwise dilution on RNA R_{T2} template. The reaction was started with 10 μ M template and at 24 h interval 1 μ L was taken out and the reaction mixture was diluted with 1 μ L of reaction buffer containing R_{L3} , R_{L4} , R_{L5} , R_{L6} (20 μ M of each), so 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 μ M dT₂₄. 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 $\pm 5\%$.

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. Control reaction of the stepwise dilution reaction. The reaction was performed in the presence of $R_{L3}, R_{L4}, R_{L5}, R_{L6}$ (20 μ M of each) only (in absence of any template) by keeping all other condition same. At 24 h interval 1 μ L was taken out and the reaction mixture was diluted with 1 μ L of reaction buffer containing $R_{L3}, R_{L4}, R_{L5}, R_{L6}$ (20 μ M 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.

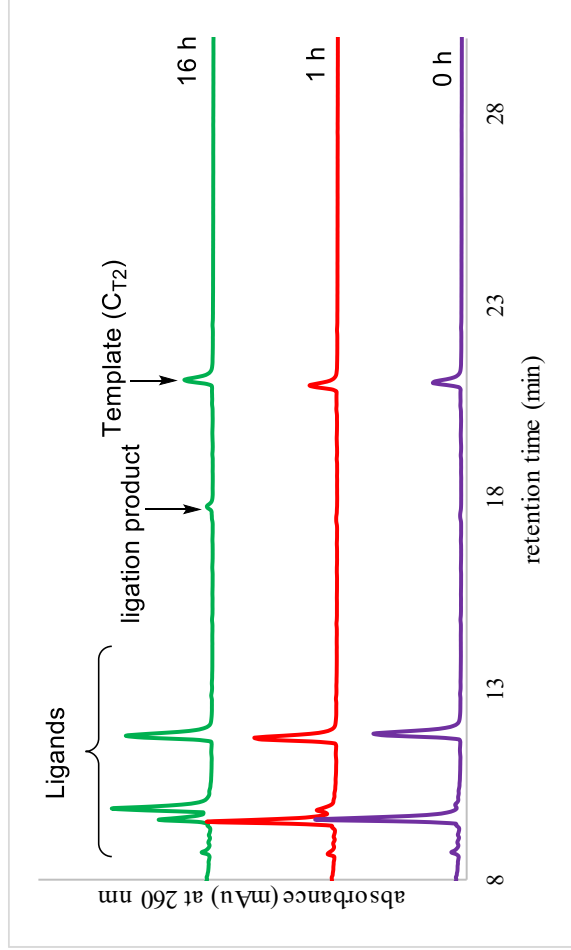
One-pot cross-catalytic replication. RNA ligands and complementary RDNA chimeric ligands on RDNA chimeric template



Entry	C _{T2} :R _{L3} ,R _{L4} (1:5)			C _{T2} :R _{L3} ,R _{L4} :C _{L7} ,C _{L8} (1:5:2)		
5'-AUUUUAAA(NH) <u>TAUUAAUUA</u> -3'	24 h	48 h	72 h	24 h	48 h	72 h
Ligation product R _{P2}	108%	110%	108%	125%	148%	160%
Yield (%)						
3'- <u>AAAAUUUA</u> (NH) <u>TAUUAAUUA</u> -5'	24 h	48 h	72 h	24 h	48 h	72 h
Regeneration of template with phosphoramidate C _{T2} ^{NH}	-	-	-	16%	36%	48%
Yield (%)						

Supplementary Figure 94: The AEC traces of the one-pot ligation reaction of R_{L3},R_{L4} (50 μM of each) and C_{L7},C_{L8} (20 μM of each) on DNA-RNA chimeric template C_{T2} (10 μM) in 0.2M EDC in 0.1M HEPES, 150 mM NaCl, pH 7.5 buffer. The AEC traces indicates the appearance of 1st ligation product (R_{P2}) and regeneration of the template with phosphoramidate linkage (C_{T2}^{NH}) (Top). The control experiment in the absence of C_{L7},C_{L8} ligands showed the leveling of the R_{P2} formation after 24 h (Bottom table) while the presence of C_{L7},C_{L8} showed the increasing formation of R_{P2}. *Gradient: 0 to 40% Buffer B in 1 min then 40 to 90% Buffer B in 30 min.*

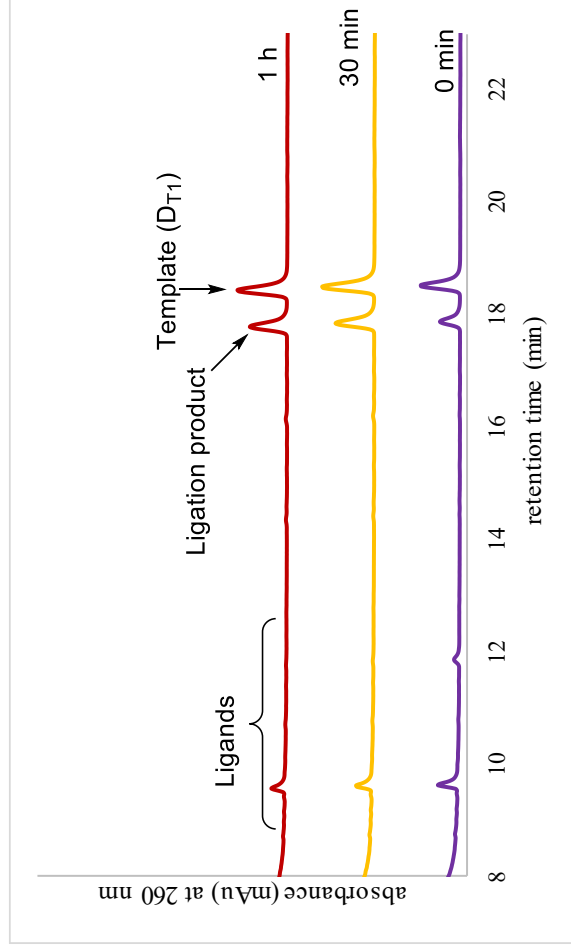
Enzymatic Ligation reaction. T4 DNA Ligase mediated ligation reaction of DNA ligands (D_{L1}, D_{L2}) on RDNA chimeric template (C_{T2})



Entry	Sequence	Expected mass	Observed mass
Ligand D_{L3}	5'- <u>TTTTTAAAT</u> ^{OH-3'}	2398	2401
Ligand D_{L2}	5'- PO_3^{2-} - <u>ATAATAAT</u> -3'	2496	2498
Template C_{T2}	3'- <u>AAAAUUUA</u> <u>UAUUUUUA</u> -5'	4893	4894
Ligation product D_{P2}	5'- <u>TTTTAAATATAATAAT</u> -3'	4877	4878
Yield (%)	1 h		
	16 h		
	<5%	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 $^\circ\text{C}$. 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.*

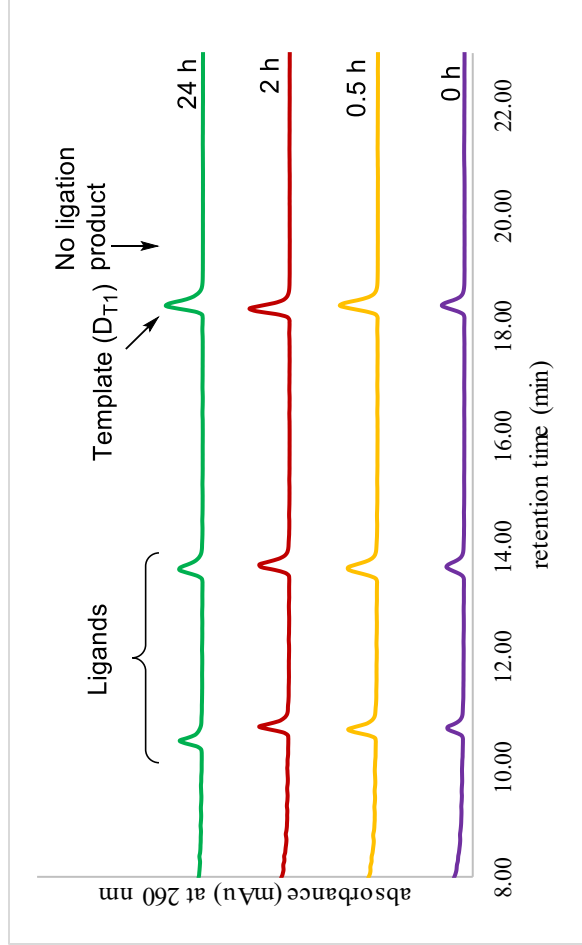
Enzymatic Ligation reaction. T4 DNA Ligase mediated ligation reaction of DNA ligands (D_{L1}, D_{L2}) on DNA template (D_{T1})



Entry	Sequence	Expected mass	Observed mass
Ligand D_{L3}	5'-TTTTTAAAT ^{OH-3'}	2398	2401
Ligand D_{L2}	5'-PO ₃ ²⁻ -ATAATAAT-3'	2496	2499
Template D_{T1}	3'-AAAAATTTATATTATTAA-5'	4877	4881
Ligation product D_{P2} Yield (%)	5'-TTTTTAAATATAATAAT-3'	4877	4881
	0 min	60 min	(both template and ligation product have same mass)
	50%	72%	74%

Supplementary Figure 96: AEC traces of the ligation reaction of DNA ligands (10 μ M of each) on DNA D_{T1} template (10 μ M) in presence of T4 DNA ligase at 4 $^\circ$ 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.*

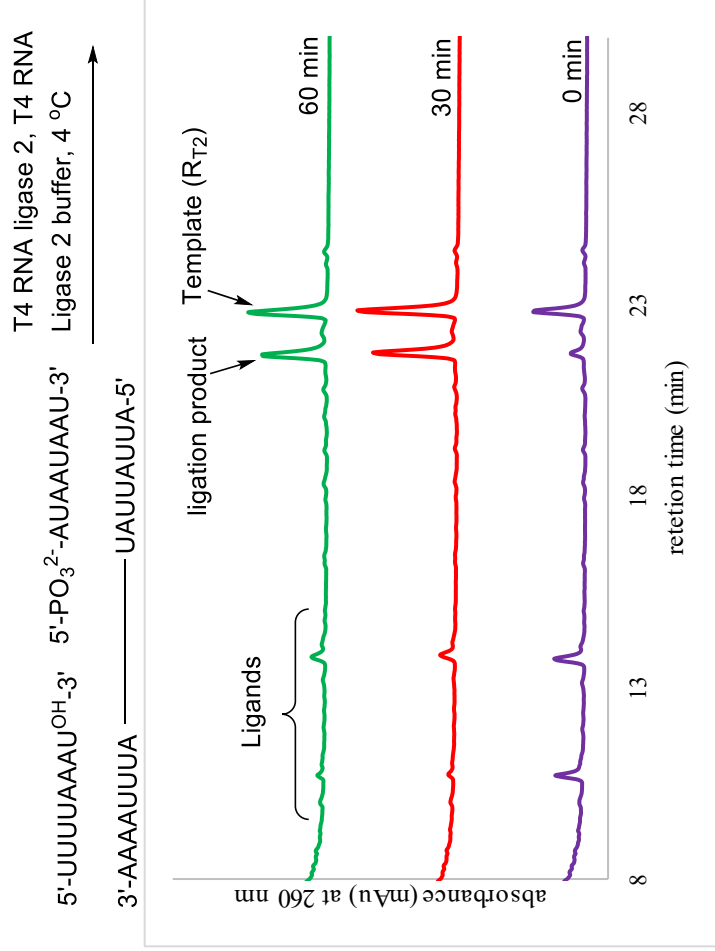
Enzymatic Ligation reaction. T4 DNA Ligase mediated ligation reaction of RNA ligands (R_{LI1}, R_{L4}) on DNA template (D_{T1})



Entry	Sequence	Expected mass	Observed mass
Ligand R _{LI1}	5'-UUUUUAAAU ^{OH-3'}	2456	2457
Ligand R _{L4}	5'-PO ₃ ²⁻ -AUAAUAAU-3'	2582	2583
Template D _{T1}	3'- <u>AAAATTTATATTATTA</u> -5'	4877	4877
Ligation product R _{P6}	5'-UUUUUAAAUAAUAAU-3'	5021	Not Observed

Supplementary Figure 97: AEC traces of the ligation reaction of RNA ligands (10 μ M of each) on DNA (D_{T1}) template (10 μ M) in presence of T4 DNA ligase at 4 $^\circ$ 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.*

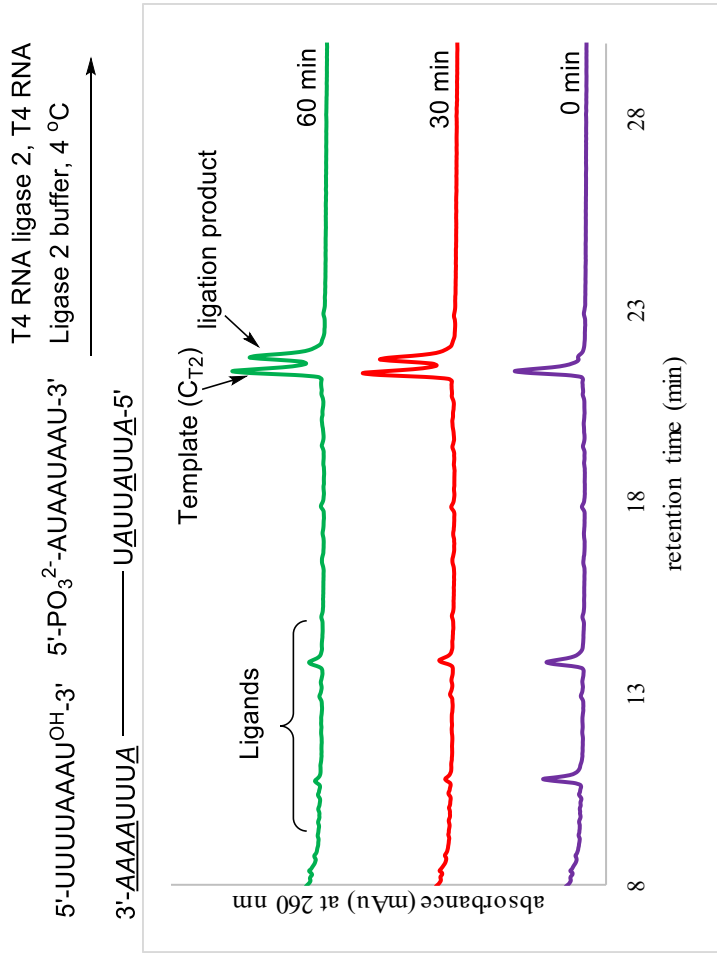
Enzymatic Ligation reaction. T4 RNA Ligase2 mediated ligation reaction of RNA ligands (R_{L11}, R_{L4}) on RNA template (R_{T2})



Entry	Sequence	Expected mass	Observed mass
Ligand R _{L11}	5'-UUUUUUUU ^{OH} -3'	2456	2458
Ligand R _{L4}	5'-PO ₃ ²⁻ -AUAAUUAAU-3'	2582	2584
Template R _{T2}	3'-AAAAUUUAUUUUUA-5'	5021	5023
Ligation product R _{P6}	5'-UUUUUUUUAAUUAAU-3'	5021	5023
Yield (%)	0 min	60 min	(both template and ligation product have same mass)
	28%	84%	87%

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.*

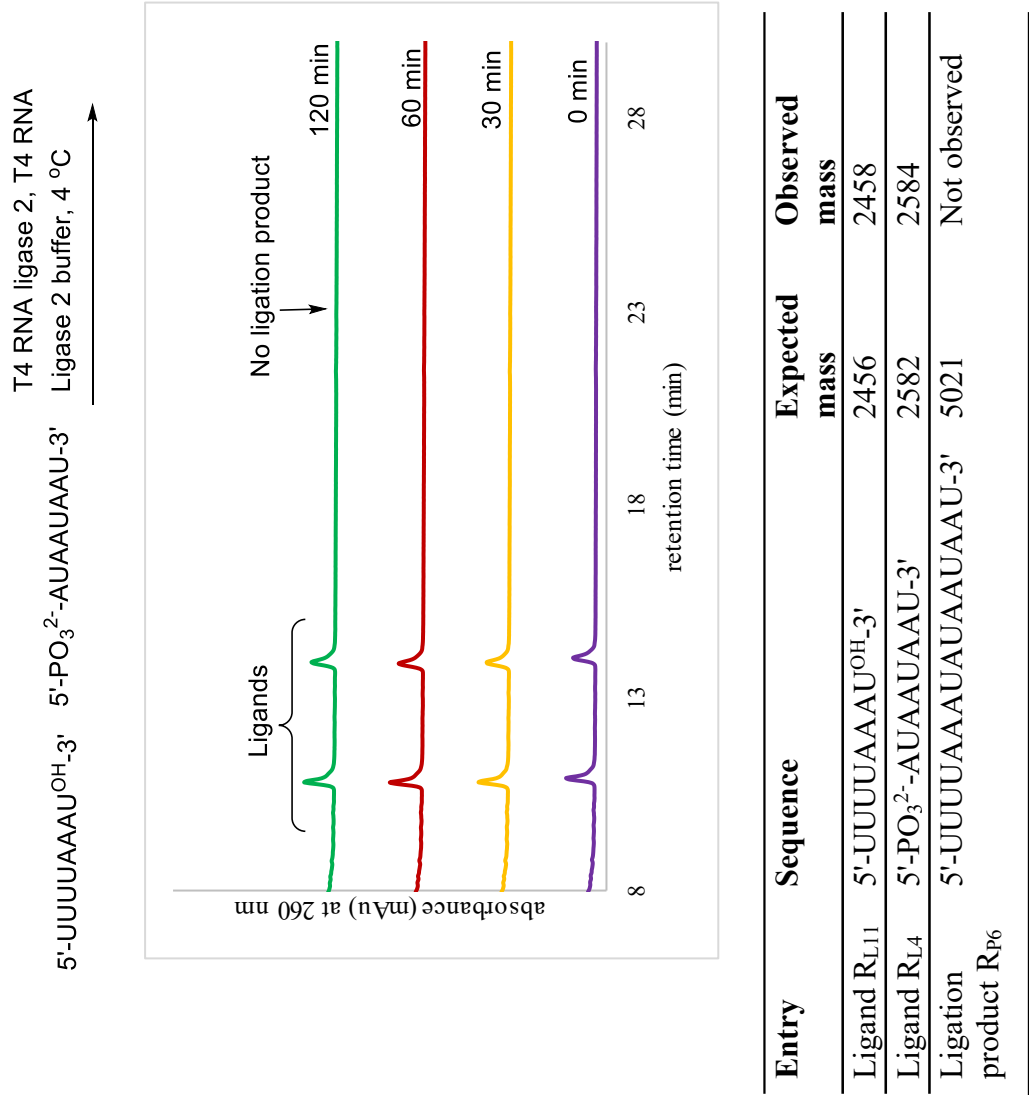
Enzymatic Ligation reaction. T4 RNA Ligase2 mediated ligation reaction of RNA ligands (R_{L11}, R_{L4}) on chimeric RDNA template (C_{T2})



Entry	Sequence	Expected mass	Observed mass
Ligand R _{L11}	5'-UUUUUUAAA U^{OH} -3'	2456	2457
Ligand R _{L4}	5'-PO ₃ ²⁻ -AUAUAUU-3'	2582	2583
Template C _{T2}	3'- <u>AAAA</u> UUU <u>AUA</u> UU <u>A</u> UU <u>A</u> -5'	4893	4893
Ligation product R _{P6}	5'-UUUUUUAAAUAUAUU-3'	5021	5021
Yield (%)	0 min 30 min 60 min		
	0% 79% 79%		

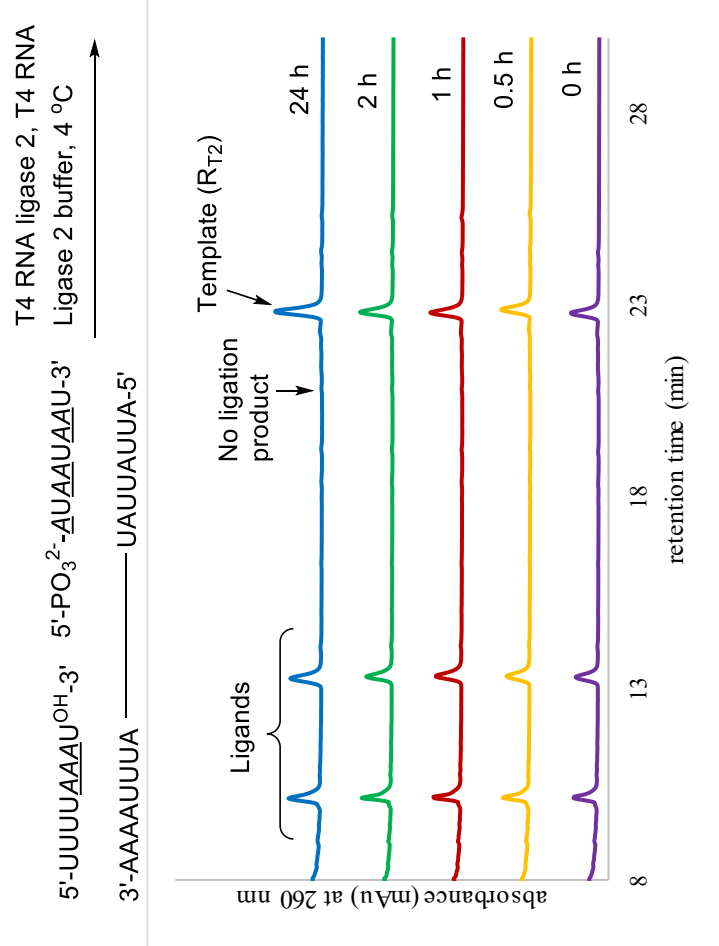
Supplementary Figure 99: AEC traces of the ligation reaction of RNA ligands (10 μM of each) on DNA-RNA chimeric (R_{T2}) template (10 μM) in presence of T4 RNA ligase 2 at 4 $^\circ\text{C}$. The peak corresponding to the ligation product was observed (Top) which was further confirmed by the MALDI-TOF 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



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.*

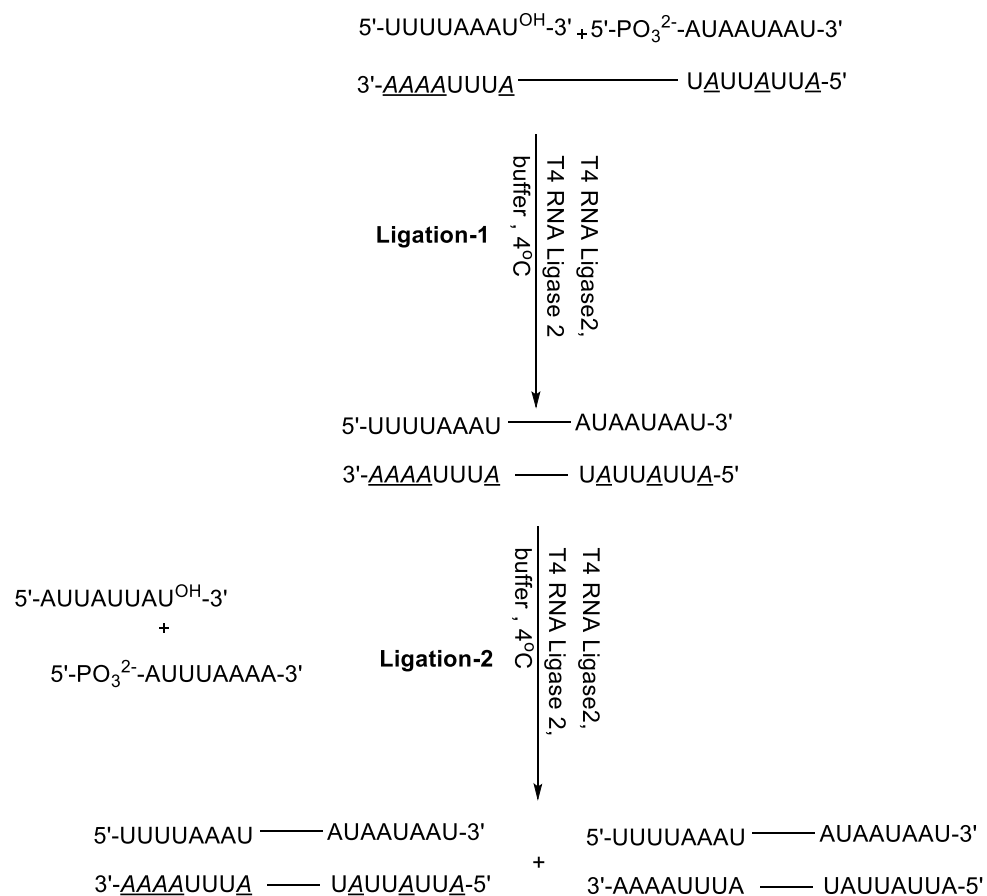
Enzymatic Ligation reaction. T4 RNA Ligase2 mediated ligation reaction of Chimeric RDNA ligands on RNA template (R_{T2})



Entry	Sequence	Expected mass	Observed mass
Ligand C _{L9}	5'-UUUU <u>AAA</u> U ^{OH} -3'	2408	2410
Ligand C _{L10}	5'-PO ₃ ²⁻ - <u>AUAAUAA</u> U-3'	2502	2504
Template R _{T2}	3'-AAAAUUUAUAUAUUUA-5'	5021	5023
Ligation product C _{P5}	5'-UUUU <u>AAA</u> U <u>AUAUAUUAA</u> U-3'	4893	Not observed

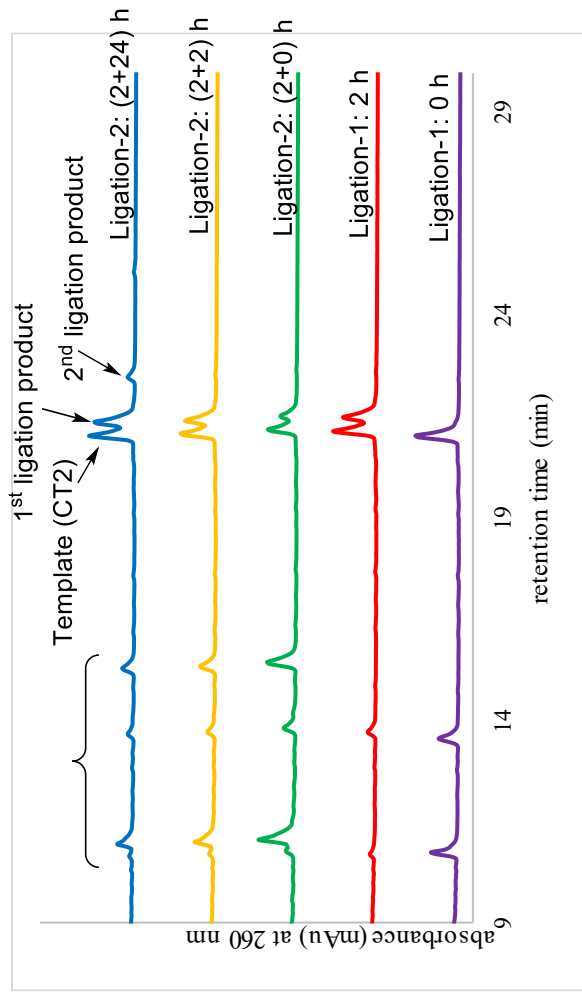
Supplementary Figure 101: AEC traces of the ligation reaction of DNA-RNA chimeric ligands (10 μM of each) on RNA (R_{T2}) template (10 μM) in presence of T4 RNA ligase 2 at 4 °C. Even after 24 h, no peak corresponding to the ligation product was observed (Top) which was further confirmed by the MALDI-TOF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

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 _{L11}	5'-UUUUAAA <u>AU</u> ^{OH} -3'
Ligand R _{L4}	5'-PO ₃ ²⁻ -AUAAUAAU-3'
Ligand R _{L12}	5'-AUUAUUAAU ^{OH} -3'
Ligand R _{L6}	5'-PO ₃ ²⁻ -AUUUAAA-3'
Template C _{T2}	3'- 444 UUU <u>4U</u> <u>4UU</u> <u>4UU</u> <u>4</u> -5'
1 st Ligation	5'-UUUUAAAUAUAAUAAU-3'
product R _{P6}	2 h 2+2 h 2+24 h
Yield (%)	79% 82% 93%
2 nd Ligation	3'-AAAAUUUAUAUUAUUA-5'
product R _{P7}	2+2 h 2+24 h
Yield (%)	<5% 18%

Supplementary Figure 103: AEC traces of the ligation reaction of the RNA ligands (10 μ M of each) on DNA-RNA chimeric (C_{T2}) template (10 μ M) 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 1st ligation product was added. But, even after 2 h, no peak corresponding to the 2nd 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 2nd 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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