

Freeze-thaw cycles as drivers of complex ribozyme assembly

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Supplementary Methods

1. Oligonucleotides

RNA and DNA oligonucleotides used in this study are described in detail in Supplementary Tab. 1 (RNA) and Supplementary Tab. 2 (DNA). The DNA templates for transcription of tC19Z gene variants and RPR7 variants were generated by PCR of gblock gene fragments (IDT). The DNA templates for all other RNAs were created by fill-in of two DNA oligonucleotides using GoTaq (Promega). DNA was purified using QiaQuick (Qiagen). RNAs were transcribed (MegaShortScript high-yield transcription kit, Ambion), and purified using RNeasy (Qiagen) or gel-purification. RNA fragments used for direct ligation by 3frHPz were ordered with 2'/3'-phosphates, which were cyclized using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma) as described before¹. All EDC treated RNAs were precipitated with isopropanol and washed extensively with 70% ethanol. Dried RNA pellets (3 – 8 nmol RNA) were resuspended in 100 μ L 1x rAPid Alkaline phosphatase buffer containing 10 U rAPid Alkaline phosphatase (Roche) and incubated for 15 min at 37°C to remove trace amounts of uncyclized phosphates. Subsequently, >p activated ligation substrates were separated from dephosphorylated oligonucleotides by urea-page and purified by gel-extraction. The 5'-labeled ligation product of RPR4 fragment 1 and 2 (Fig. 3e) was prepared by gel-purification from a large-scale ligation reaction described below. All oligonucleotide concentrations were determined spectroscopically using the extinction coefficients at 260 nm calculated by OligoCalc (<http://www.basic.northwestern.edu/biotools/oligocalc.html>)²

1. Analysis of FT-driven ligation

Fractional fluorescence intensities of individual ligation products were fitted assuming that fragment ligations occurred independently from each other and that the fraction of unreacted HPz-substrate complexes remained constant during each cycle. The increase in relative ligation (l) of two fragments in relation to the number n of cycles can be estimated by

$$l(n) = L_{\max}(1 - f_u^n) \quad (\text{S1})$$

where f_u is the fraction of product that remains unligated during each cycle and $L_{\max}$ is the maximal ligation level of that ligation site that could be achieved after an infinite number of cycles. Relative yields of 5'-fragments that have been extended in 3' direction by ligation of k segments are given by

$$p_{[1,k]}(n) = (1 - l_k(n)) \cdot \prod_{i=1}^{k-1} l_i(n) \quad (\text{S2})$$

where $l_k(n)$ is the fraction of segment $k-1$ that has been ligated to segment k after n cycles. For values of k exceeding the number of the last ligatable 3'-fragment $l_k(n)$ is defined as zero. Best-fit global parameters for L_{max} and f_u of each ligation site were obtained by parallel least-square fitting of every $p_{[1,k]}(n)$ to the respective lane percentages of its corresponding ligation product ($p_{[1,1]}(n)$ to $p_{[1,7]}(n)$ for RPR7).

3. Primer extension assays

Full-length RPR or RPR fragments were annealed with FITC- or Alexa647-PRIR1 and Temp1 (60°C for 1 s, 0.1°C/s to 16°C, 16°C for 10 min), chilled on ice, and mixed with chilled reaction buffer (final concentration 200 mM MgCl₂, 4 mM NTPs, 50 mM Tris•HCl pH 8.3/25°C). Primer extensions were carried out at 17°C. Final RNA concentrations during primer extension were 0.5 μM. Unless stated otherwise, reactions were stopped after 96 hours by mixing with reactions with a 4-fold excess of quenching buffer (8.625 M urea, 55 mM EDTA/pH 8.0, 1.25 μM CompTemp1). All samples were resolved by urea-PAGE of 0.5 pmol fluorescently labelled primer and analysed using a Typhoon Trio scanner (GE Healthcare). Amounts of primer extension were quantified as described previously³.

Primer extension reactions of ligation mixtures were performed by taking samples of stoichiometric ligation samples (1 μM fragment concentrations) and mixing them with Alexa467-PRIR, Temp 1, and a 1.76-fold molar excess of SBS antisense oligonucleotides (Supplementary Table S1). After annealing, chilled reaction buffer was added to the samples (final fragment, template and primer concentrations were 0.5 μM) and reactions were incubated and analysed as described above.

4. Selection of additional HPz SBS/substrate combinations

The HPrz selection library with randomized nucleotides in the substrate, SBS, and loop B motifs was produced by *in vitro* transcription from its DNA template (MegaShortScript high-yield transcription kit, Ambion) followed by TURBO DNase (Life Technologies) treatment and RNeasy (Qiagen) purification. Eluted RNA was 5' dephosphorylated using rAPid alkaline phosphatase (Roche) followed by a second RNeasy purification step. Selection of non-natural SBS-substrate combinations was performed by mixing HPrz_lib RNA (60 pmol in round 1, 30 pmol for round 2/3, final concentration 1.55 μM) with HPrz_bait (66 pmol in round 1, 33 pmol for round 2/3 final concentration 1.7 μM) in standard HPz ligation buffer (1 mM Tris•HCl pH 8.3/25°C, 25 mM NaCl). Following incubation at 37°C for 15 min, samples were frozen and incubated at -9°C for 3 h. Self-ligated ribozymes were captured by adding 700 μg Dynabeads MyOne Streptavidin C1 (Life Technologies) in 250 μL BWBT (0.2 M NaCl, 10 mM Tris•HCl, pH 7.4, 1 mM EDTA, 0.1% Tween-20). After incubation at room temperature for 30 min, the beads were extracted using a magnetic separation stand (Promega) and washed with BWBT, 30% formamide and ddH₂O. Next, all supernatant was removed and beads were resuspended

in 200 μ L 1x Superscript III RT-PCR reaction mix (Life Technologies) containing 0.2 μ M of HPrz_sel_fw and HPrz_sel_rev followed by first-strand synthesis at 50°C for 20 min. RNA was removed by adding 25 U RNase H (NEB, 37°C for 20 min). Magnetic beads were removed prior to polyclonal cDNA amplification (18 cycles of 94°C for 30 s, 48°C for 30 s, 68°C for 20 s; 12 cycles for all other selection rounds). The PCR product was purified (Qiaquick PCR purification, Qiagen) and used to transcribe RNA for the next round of selection.

5. Deep sequencing

Amplified polyclonal cDNA from HPz selections was prepared for deep sequencing by the Illumina Miseq method by appending the bridge-amplification sequences by PCR. Sequencing library generating PCR reactions were performed with OneTaq Hot Start master mix (Promega) using 0.1 μ M primers and 50 ng cDNA (16 cycles of 94°C for 30 s, 48°C for 30 s, 68°C for 20 s). PCR products were gel-purified and 12 pmol of pooled libraries plus 20% PhiX control (Illumina) were denatured and sequenced (single-end read, 75 cycles) using a MiSeq reagent kit and instrument (Illumina) according to manufacturer's instructions. Libraries were barcoded using P5 primers containing 6 nts sequences from the NEXTflex series (Illumina). Data were filtered, trimmed, and collapsed using the FastaX command line toolkit (http://hannonlab.cshl.edu/fastx_toolkit/commandline.html). For each of the 64 selected substrate sequences, sequence logos of the substrate binding strand were created using Jalview⁴. Randomized regions of loop B mostly converged to the wild type consensus sequence and were not further analysed in this study.

Supplementary Table 1 | RNA oligonucleotides used in this study. IVT = in vitro transcription; EDC = treated with EDC to generate >p from oligonucleotides ending in 3'phosphate.

Name	Source	Sequence (5' to 3')	Description
F24MM_fw	IDT	5TEX615-GAUUAUCACACGGCACACUUAUAG	5' Texas 615 labelled strand of the internal loop used for strand invasion experiments.
24_fw	IDT	GAUUAUCACACGGCACACUUAUAG	unlabelled strand of the internal loop used for strand invasion experiments.
24Q_rev	IDT	CUAUAAGUGUCGGCUGUGAUAAUC-3IAbRQSp	3' Iowa Black RQ labelled strand of the internal loop used for strand invasion experiments.
loopB5	IDT	ACGUGAGAAACACUGCAG	5' fragment of 3frHPz loop B used in all ligation experiments
loopB3	IDT	CUGCAGGUACAUUACCACGUA	3' fragment of 3frHPz loop B used in all ligation experiments
SBS.16mer	Sigma	GGGCAGAGAAGUCA	SBS used for concatenation of 16mer repeats
16mer	IDT, EDC	GUCCUGCCCAAUGACA>p	>p activated 16-mer repeat substrate used for model concatenations.
FRep16mer	IDT, EDC	6FAM-CAGUCUAGCAAGUAUGCGUCUGACAGUCCUGC CCAAUGACA>p	6-carboxyfluorescein labelled reporter RNA used in model concatenations. The RNA contains an internal HPz site to monitor cleavage in parallel to ligation
RPR4.1	IDT, EDC	6FAM-GUCAUUGAAAAAAGACAAAUCUGCCCUCA GAGCUUGAGAACACA>p	>p activated RPR4 fragment 1 with a 5' 6-carboxyfluorescein used for direct ligation
RPR4.2	IDT, EDC	GUCGUGUCAGAGGAGGCAGCCUUCGGUGGCGCGAUA GCGCCAACGUUCUCAACA>p	>p activated RPR4 fragment 2 used for direct ligation
RPR4.3	Sigma, EDC	GUCGCCCCAAUACUCCCGCUUCGGCGGGUGGGGAUAC A>p	>p activated RPR4 fragment 3 used for direct ligation
RPR4.4	IDT	GUCGACGAAAAGGCGAUGUAGACACGCCCGACUCAU AAUCCCCGGAGCUUCGGCUCC	Fragment 3 of RPR4 used for direct ligation and for assembly from inert oligonucleotides.
RPR4.1t	IDT	6FAM-GUCAUUGAAAAAAGACAAAUCUGCCCUCA GAGCUUGAGAACACA GUCGUGUG	RPR4 fragment 1 with cleavable 8 nts 3' tail (green) used for assembly from inert oligonucleotides. Contains a 5' 6-carboxy-fluorescein
RPR4.2t	IVT	GUCGUGUCAGAGGAGGCAGCCUUCGGUGGCGCGAUA GCGCCAACGUUCUCAACA GUCGCCC	RPR4 fragment 2 with cleavable 7 nts 3' tail (green)
RPR4.3t	IVT	GUCGCCCCAAUACUCCCGCUUCGGCGGGUGGGGAUAC A GUCGACGA	RPR4 fragment 3 with cleavable 8 nts 3' tail (green)
RPR4.12	IVT	GUCAUUGAAAAAAGACAAAUCUGCCCUAGAGCU UGAGAACACAGUCGUGUCAGAGGAGGCAGCCUUCGG UGGCGCGAUAGCGCCAACGUUCUCAACA	Product formed by ligation of RPR4 fragment 1 and 2
RPR4.123	IVT	GUCAUUGAAAAAAGACAAAUCUGCCCUAGAGCU UGAGAACACAGUCGUGUCAGAGGAGGCAGCCUUCGG UGGCGCGAUAGCGCCAACGUUCUCAACAGUCGCCAA UACUCCCGCUUCGGCGGGUGGGGAUAACA	Product formed by ligation of RPR4 fragment 1, 2, and 3.
RPR4.23	IVT	GUCGUGUCAGAGGAGGCAGCCUUCGGUGGCGCGAUA GCGCCAACGUUCUCAACAGUCGCCCAAUACUCCCGCU UCGGCGGGUGGGGAUAACA	Product formed by ligation of RPR4 fragment 2 and 3.
RPR4.34	IVT	GUCGCCCCAAUACUCCCGCUUCGGCGGGUGGGGAUAC AGUCGACGAAAAGGCGAUGUAGACACGCCCGACUCA UAAUCCCCGGAGCUUCGGCUCC	Product formed by ligation of RPR4 fragment 3 and 4.
RPR4.FL	IVT	GUCAUUGAAAAAAGACAAAUCUGCCCUAGAGCU UGAGAACAC AGUCGUG UCAGAGGAGGCAGCCUUCGG UGGCGCGAUAGCGCCAACGUUCUCAAC AGUCGCCAA UACUCCCGCUUCGGCGGGUGGGGAUAACA AGUCGACGA AAAGGCGAUGUAGACACGCCCG GAC UCAUAAUCCCG GAGCUUCGGCUCC	Full-length RPR4. Sequence deviations from tC19Z are highlighted in red.
SBS.RPR4.1	Sigma	CUGCACACAGAAGUGUUCUC	3frHPz SBS used for ligation of RPR4 fragment 1 and 2

SBS.RPR4.2	Sigma	AUUGGGCAGAAGUUGAGAAC	3fHPz SBS used for ligation of RPR4 fragment 2 and 3
SBS.RPR4.3	Sigma	CUUUUCGUCAGAAGUUAUCCC	3fHPz SBS used for ligation of RPR4 fragment 3 and 4
SBS.RPR4.1as	Sigma	GAGAACACUUCUGUGUGCAG	Antisense strand for SBS.RPR4.1 to quench HPz catalysis during primer extension reactions
SBS.RPR4.1as	Sigma	GUUCUCAACUUCUGCCCAAU	Antisense strand for SBS.RPR4.2 to quench HPz catalysis during primer extension reactions
SBS.RPR4.1as	Sigma	GGGAUAACUUCUGACGAAAAG	Antisense strand for SBS.RPR4.3 to quench HPz catalysis during primer extension reactions
RPR7.1	IDT, EDC	6FAM-GUCAUUGAAAAAAGACAAAUCUGCCCUCA>p	>p activated RPR7 fragment 1 with a 5' 6-carboxyfluorescein
RPR7.2	IDT, EDC	GAGCUUGAGAAACUUCUGGAUGCA>p	>p activated RPR7 fragment 2
RPR7.3	IDT, EDC	GAGGAGGCAGCCUUCGGUGGCGCGACA>p	>p activated RPR7 fragment 3
RPR7.4	IDT, EDC	GCGCCAACGUUCUCAACA>p	>p activated RPR7 fragment 4
RPR7.5	IDT, EDC	GUCGCCCCAAUACUCCCGCUUCGGCGGGCG>p	>p activated RPR7 fragment 5
RPR7.6	IDT, EDC	GGGAUAACACCUAGACGAAAAGCG>p	>p activated RPR7 fragment 6
RPR7.7	IDT	GAUGUUAAGACACCGCCAGGUCAUAAUCCCC	RPR7 fragment 7
RPR7.FL	IVT	GUCAUUGAAAAAAGACAAAUCUGCCCUAGAGCUUG AGAACAUCUUCGGAUGCAGAGGAGGCAGCCUUCGGUG GCGCGACAGCGCCAACGUUCUCAACAGUCGCCCAAUA CUCCCGCUUCGGCGGGCGGGGAUAACACCUAGACGAAA AGCGGAUGUUAGACACCGCCAGGUCAUAAUCCCC	Full-length RPR7
SBS.RPR7.1	IDT	UUCUCAAGCGAAGAGGGCAG	3fHPz SBS used for ligation of RPR7 fragment 1 and 2
SBS.RPR7.2	IDT	CUGCCUCUGAAGCAUCCGAA	3fHPz SBS used for ligation of RPR7 fragment 2 and 3
SBS.RPR7.3	IDT	ACGUUGGUGAAGUCGCGCCA	3fHPz SBS used for ligation of RPR7 fragment 3 and 4
SBS.RPR7.4	IDT	GUAUUGGGCAGAAGUUGAGA	3fHPz SBS used for ligation of RPR7 fragment 4 and 5
SBS.RPR7.5	IDT	GGUGUUAUCGAAGCCCGCCGAAG	3fHPz SBS used for ligation of RPR7 fragment 5 and 6
SBS.RPR7.6	IDT	GUCUAACGGAAGCUUUCGUC	3fHPz SBS used for ligation of RPR7 fragment 6 and 7
FITC-PRIR1	Sigma	FITC-GAAUCAAGGG	5' Fluorescein isothiocyanate labelled primer for primer extension assays
Alexa647-PRIR1	IDT	Alexa647-GAAUCAAGGG	5' Alexa647 labelled primer for primer extension assays in presence of fluorescein-labelled RPR fragments
Temp1	IVT	GACAUGACAUAUAAACAGAUUGGACCUCGG <u>CCCUUGA</u> <u>UUC</u> AAAAAAAAA	Model template used to monitor RPR primer extension activity. The primer-binding site is highlighted in yellow. The C19 site for RPR tethering ⁵ is underlined
CompTemp1		UUUUUUUUUAAUCAAAGGGCCGAGGUCCAUCUGUU UAAUGUCAUUGUC	Competing oligonucleotide to facilitate release of PRIR1 from Temp1 during gel electrophoresis
tC19Z	IVT	GUCAUUGAAAAAAGACAAAUCUGCCCUAGAGCU UGAGAACAUCUUCGGAUGCAGAGGAGGCAGCCUUCGG UGGCGCGAUAGCGCCAACGUUCUCAACAGACACCCAA UACUCCCGCUUCGGCGGGUGGGGAUAACACCUAGACGA AAAGGCGAUGUUAGACACGCCCAGGUCAUAAUCCCCG GAGCUUCGGCUCC	"wild type" tC19Z
tC19Z_9	IVT	GUCAUUGAAAAAAGACAAUUCUGCCCUAGAGCU UGAGAACAUCUUCGGAUGCAGAGGAGGCAGCCUUCGG UGGCGCGAUAGCGCCAACGUUCUCAACAGACACCCAA UACUCCCGCUUCGGCGGGUGGGGAUAACACCUAGACGA AAAGGCGAUGUUAGACACGCCCAGGUCAUAAUCCCCG GAGCUUCGGCUCC	tC19Z with cognate HPz substrate site at pos 9. Numbering of the ligation site is according to original R18 RPR ⁶ .
tC19Z_23	IVT	GUCAUUGAAAAAAGACAAAUCUGCCCUACAGUU UGAGAACAUCUUCGGAUGCAGAGGAGGCAGCCUUCGG UGGCGCGAUAGCGCCAACGUUCUCAACAGACACCCAA UACUCCCGCUUCGGCGGGUGGGGAUAACACCUAGACGA AAAGGCGAUGUUAGACACGCCCAGGUCAUAAUCCCCG	tC19Z with cognate HPz substrate site at pos 23 (numbering according to the original R18 RPR)

		GAGCUUCGGCUCC	
tC19Z_36	IVT	GUCAUUGAAAAAAAAGACAAAUCUGCCCUAGAGCU UGAGAACA CAGUCGUG UGCAGAGGAGGCAGCCUUCGG UGGCGCGAUAGCGCCAACGUUCUCAACAGACACCCAA UACUCCCGCUUCGGCGGGUGGGGAUAACACCUGACGA AAAGGCGAUGUUAGACACGCCCAGGUCAUAAUCCCG GAGCUUCGGCUCC	tC19Z with cognate HPz substrate site at pos 36 (numbering according to the original R18 RPR ⁶)
tC19Z_46	IVT	GUCAUUGAAAAAAAAGACAAAUCUGCCCUAGAGCU UGAGAACAUCUUCGGAUGCAG UCGAGGCAGC GAUCGG UGGCGCGAUAGCGCCAACGUUCUCAACAGACACCCAA UACUCCCGCUUCGGCGGGUGGGGAUAACACCUGACGA AAAGGCGAUGUUAGACACGCCCAGGUCAUAAUCCCG GAGCUUCGGCUCC	tC19Z with cognate HPz substrate site at pos 46 (numbering according to the original R18 RPR ⁶)
tC19Z_55	IVT	GUCAUUGAAAAAAAAGACAAAUCUGCCCUAGAGCU UGAGAACAUCUUCGGAUGCAGAG AAGGCAGU CUUCGG UGGCGCGAUAGCGCCAACGUUCUCAACAGACACCCAA UACUCCCGCUUCGGCGGGUGGGGAUAACACCUGACGA AAAGGCGAUGUUAGACACGCCCAGGUCAUAAUCCCG GAGCUUCGGCUCC	tC19Z with cognate HPz substrate site at pos 55 (numbering according to the original R18 RPR ⁶)
tC19Z_73	IVT	GUCAUUGAAAAAAAAGACAAAUCUGCCCUAGAGCU UGAGAACAUCUUCGGAUGCAGAGGAGGCAGCCUUCGG UGGGA CGAUAG UCC AACGUUCUCAACAGACACCCAA UACUCCCGCUUCGGCGGGUGGGGAUAACACCUGACGA AAAGGCGAUGUUAGACACGCCCAGGUCAUAAUCCCG GAGCUUCGGCUCC	tC19Z with cognate HPz substrate site at pos 73 (numbering according to the original R18 RPR ⁶)
tC19Z_81	IVT	GUCAUUGAAAAAAAAGACAAAUCUGCCCUAGAGCU UGAGAACAUCUUCGGAUGCAGAGGAGGCAGCCUUC UG UGGCGCGAUAGCGCCA UA GUUCUCAACAGACACCCAA UACUCCCGCUUCGGCGGGUGGGGAUAACACCUGACGA AAAGGCGAUGUUAGACACGCCCAGGUCAUAAUCCCG GAGCUUCGGCUCC	tC19Z with cognate HPz substrate site at pos 81 (numbering according to the original R18 RPR ⁶)
tC19Z_91C	IVT	GUCAUUGAAAAAAAAGACAAAUCUGCCCUAGAGCU UGAGAACAUCUUCGGAUGCAGAGGAGGCAGCCUUCGG UGGCGCGAUAGCGCCAACGUUCUCAACAG UCC CCAA UACUCCCGCUUCGGCGGGUGGGGAUAACACCUGACGA AAAGGCGAUGUUAGACACGCCCAGGUCAUAAUCCCG GAGCUUCGGCUCC	tC19Z with cognate HPz substrate site at pos 91, variant 1 (numbering according to the original R18 RPR ⁶)
tC19Z_91G	IVT	GUCAUUGAAAAAAAAGACAAAUCUGCCCUAGAGCU UGAGAACAUCUUCGGAUGCAGAGGAGGCAGCCUUCGG UGGCGCGAUAGCGCCAACGUUCUCAACAG UCG CCAA UACUCCCGCUUCGGCGGGUGGGGAUAACACCUGACGA AAAGGCGAUGUUAGACACGCCCAGGUCAUAAUCCCG GAGCUUCGGCUCC	tC19Z with cognate HPz substrate site at pos 91, variant 2 (numbering according to the original R18 RPR ⁶)
tC19Z_91U	IVT	GUCAUUGAAAAAAAAGACAAAUCUGCCCUAGAGCU UGAGAACAUCUUCGGAUGCAGAGGAGGCAGCCUUCGG UGGCGCGAUAGCGCCAACGUUCUCAACAG UCU CCAA UACUCCCGCUUCGGCGGGUGGGGAUAACACCUGACGA AAAGGCGAUGUUAGACACGCCCAGGUCAUAAUCCCG GAGCUUCGGCUCC	tC19Z with cognate HPz substrate site at pos 91, variant 3 (numbering according to the original R18 RPR ⁶)

tC19Z_102	IVT	GUCAUUGAAAAAAAAGACAAAUCUGCCUCAGAGCU UGAGAACAUUCUGGAUGCAGAGGAGGCAGCCUUCGG UGGCGCGAUAGCGCCAACGUUCUCAACAGACACCCAA UA <u>G</u> UCCCGCUUCGGCGGGUGGGGAUAACACCUGACGA AAAGGCGAUGUUAGACACGCCCAGGUCAUAAUCCCG GAGCUUCGGCUCC	tC19Z with cognate HPz substrate site at pos 102 (numbering according to the original R18 RPR ⁶)
tC19Z_109	IVT	GUCAUUGAAAAAAAAGACAAAUCUGCCUCAGAGCU UGAGAACAUUCUGGAUGCAGAGGAGGCAGCCUUCGG UGGCGCGAUAGCGCCAACGUUCUCAACAGACACCCAA UACUCC <u>ACAGUCGUG</u> UGGGGAUAACAGCUGACGA AAAGGCGAUGUUAGACACGCCCAGGUCAUAAUCCCG GAGCUUCGGCUCC	tC19Z with cognate HPz substrate site at pos 109 (numbering according to the original R18 RPR ⁶)
tC19Z_129	IVT	GUCAUUGAAAAAAAAGACAAAUCUGCCUCAGAGCU UGAGAACAUUCUGGAUGCAGAGGAGGCAGCCUUCGG UGGCGCGAUAGCGCCAACGUUCUCAACAGACACCCAA UACUCCCGCUUCGGCGGGUGGGGAUAACA <u>GUC</u> GACGA AAAGGCGAUGUUAGACACGCCCG <u>AC</u> UCAUAAUCCCG GAGCUUCGGCUCC	tC19Z with cognate HPz substrate site at pos 129 (numbering according to the original R18 RPR ⁶)
tC19Z_160	IVT	GUCAUUGAAAAAAAAGACAAAUCUGCCUCAGAGCU UGAGAACAUUCUGGAUGCAGAGGAGGCAGCCUUCGG UGGCGCGAUAGCGCCAACGUUCUCAACAGACACCCAA UACUCCCGCUUCGGCGGGUGGGGAUAAC <u>GA</u> CUGACGA AAACGCGAUGUUAGACACGCG <u>CAGU</u> CCAUAUCCCG GAGCUUCGGCUCC	tC19Z with cognate HPz substrate site at pos 160 (numbering according to the original R18 RPR ⁶)
HPrz_lib	IVT	GNNNUGCCCUUCGGGGCANNGNNGUAGACCAGNNNN CNCACGACGUAAGUCGUGNNANNNNNCCUGGUACCCU GAGAAUUCGU	Round 0 library construct used for selection of self-ligating hairpin ribozymes
HPrz_bait	IDT, EDC	Biotin-biotin-Fluorescein_dT-CCCGCGAA AUUAAUACGACUCACUAUA>p	>p activated, 5' doubly biotinylated and fluorescein labelled bait used during HPz selection

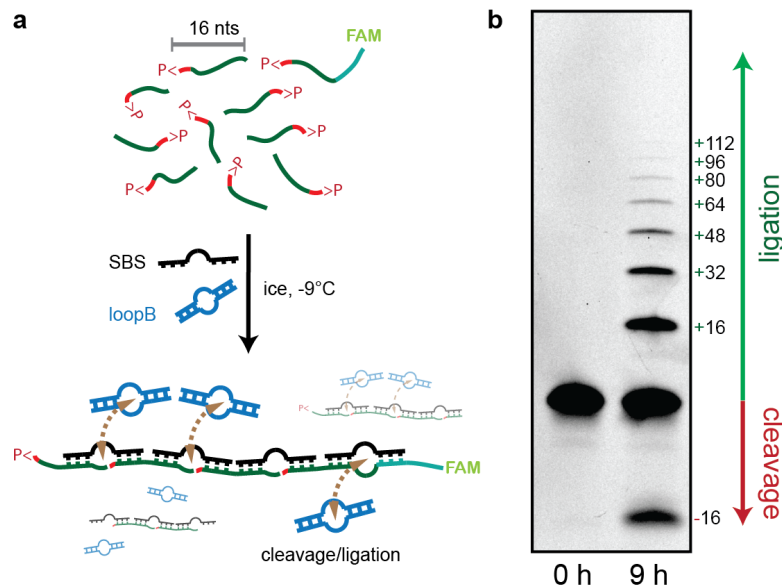
Supplementary Table 2 | DNA oligonucleotides used in this study.

Name	Source	Sequence (5' to 3')	Description
5T7	Sigma	GATCGATCTCGCCCGCGAAATTAATACGACTCACTATA	Primer encoding T7 promoter used for fill-in reactions gblock amplifications producing various IVT templates.
5T7_RPR	IDT	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAGACAAATCTGCCCTC	Primer encoding T7 promoter and parts of RPR4. Used for fill-in reactions producing the RPR4 IVT template.
RPR4.FL_FI	IDT	GGAGCCGAAGCTCCGGGGATTATGAGTCGGGCGTGTC TAACATCGCCTTTTCGTCGACTGTTATCCCCACCCGC CGAAGCGGGAGTATTGGGCGACTGTTGAGAACGTTGG CGCTATCGCGCCACCGAA	Used for fill-in reactions with 5T7_RPR4 producing the full-length RPR4 IVT template
RPR4.2t_FI	IDT	GGGCGACTGTTGAGAACGTTGGCGCTATCGCGCCACC GAAGGCTGCCCTCCTCTGCACACGACTATAGTGAGTCG TATTAATTTTC	Used for fill-in reactions with 5T7 producing the RPR4.2t IVT template
RPR4.3t_FI	IDT	TCGTCGACTGTTATCCCCACCCGCCGAAGCGGGAGTA TTGGGCGACTATAGTGAGTCGTATTAATTTTC	Used for fill-in reactions with 5T7 producing the RPR4.3t IVT template
RPR4.12_FI	IDT	TGTTGAGAACGTTGGCGCTATCGCGCCACCGAAGGCT GCCTCCTCTGCACACGACTGTTTCTCAAGCTCTGAG GGCAGATTTGTCTTTTTTTTCAATGACTATAGTGAG TCGTATTAATTTTC	Used for fill-in reactions with 5T7 producing the RPR4.12 IVT template
RPR4.123_FI	IDT	TGTTATCCCCACCCGCCGAAGCGGGAGTATTGGGCGA CTGTTGAGAACGTTGGCGCTATCGCGCCACCGAAGGC TGCCTCCTCTGCACACGACTGTGTTCTCAAGCTCTGA GGGCAGATTTGTCTTTTTTTTCAATGACTATAGTGA GTCGTATTAATTTTC	Used for fill-in reactions with 5T7 producing the RPR4.123 IVT template
RPR4.23_FI	IDT	TGTTATCCCCACCCGCCGAAGCGGGAGTATTGGGCGA CTGTTGAGAACGTTGGCGCTATCGCGCCACCGAAGGC TGCCTCCTCTGCACACGACTATAGTGAGTCGTATTAA TTTC	Used for fill-in reactions with 5T7 producing the RPR4.23 IVT template
RPR4.34_FI	IDT	GGAGCCGAAGCTCCGGGGATTATGAGTCGGGCGTGTC TAACATCGCCTTTTCGTCGACTGTTATCCCCACCCGC CGAAGCGGGAGTATTGGGCGACTATAGTGAGTCGTAT TAATTTTC	Used for fill-in reactions with 5T7 producing the RPR4.34 IVT template
RPR7.FL_IVT	IDT	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAGACAAATCTGCCCTCAGAGCTT GAGAACATCTTCGGATGCAGAGGAGGCAGCCTTCGGT GGCGGACAGCGCCAACGTTCTCAACAGTCGCCCAAT ACTCCCGCTTCGGCGGGCGGGGATAACACCTGACGAA AAGCGGATGTTAGACACCGCCAGGTCATAATCCCC	gblock for the RPR7 IVT template.
Temp1_FI	IDT	TTTTTTTTTTGAATCAAGGGCCGAGGTCCAATCTGTT TAATGTCATTGTCTATAGTGAGTCGTATTAATTTTC	Used for fill-in reactions with 5T7 producing the Temp1 IVT template
tC19Z_IVT	-	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAGACAAATCTGCCCTCAGAGC TTGAGAACATCTTCGGATGCAGAGGAGGCAGCCTTCG GTGGCGGATAGCGCCAACGTTCTCAACAGACACCCA ATACTCCCGCTTCGGCGGGTGGGGATAACACCTGACG AAAAGCGGATGTTAGACACGCCAGGTCATAATCCCC GGAGCTTCGGCTCC	Template used for IVT of tC19Z. available in the lab.

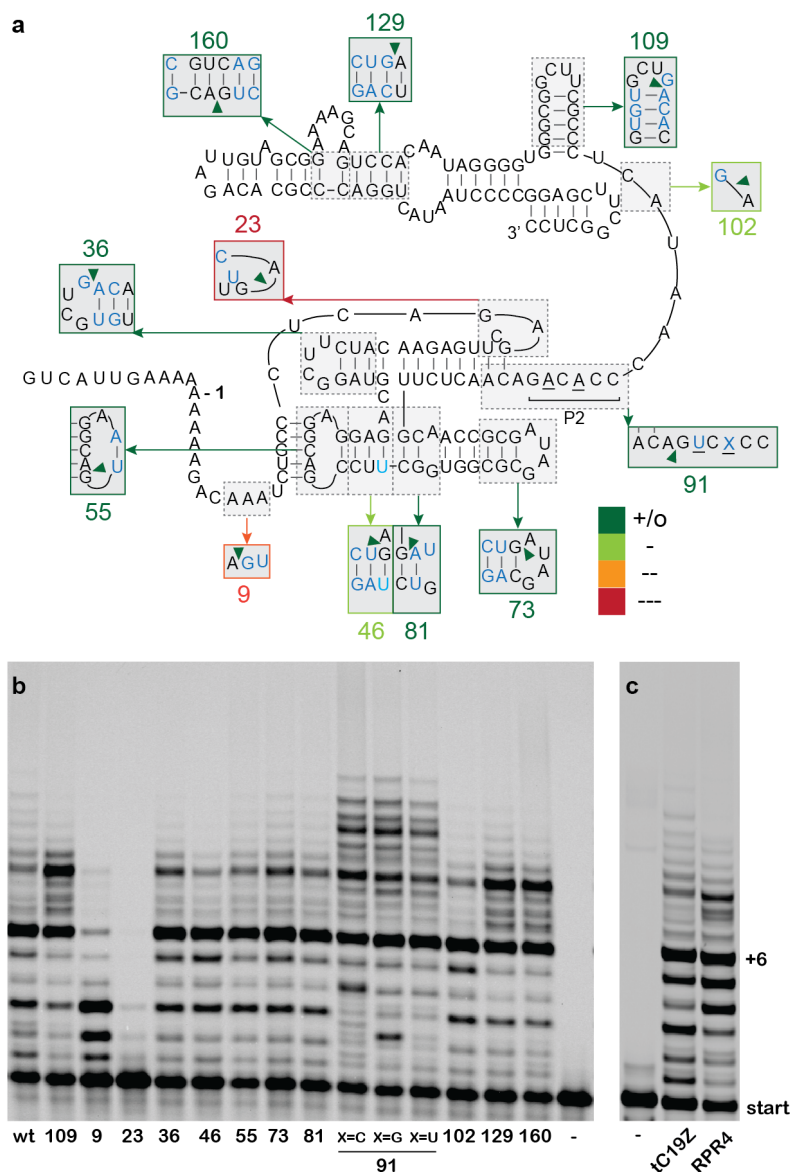
tC19Z_9_IVT	IDT, gblock	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAAAAGACAGTTCTGCCCTCAGAGC TTGAGAACATCTTCGGATGCAGAGGAGGCAGCCTTCG GTGGCGCGATAGCGCCAACGTTCTCAACAGACACCCA ATACTCCCGCTTCGGCGGGTGGGGATAACACCTGACG AAAAGGCGATGTTAGACACGCCAGGTCATAATCCCC GGAGCTTCGGCTCC	Template used for IVT
tC19Z_23_IVT	IDT, gblock	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAAAAGACAAATCTGCCCTCAGAGT TTGAGAACATCTTCGGATGCAGAGGAGGCAGCCTTCG GTGGCGCGATAGCGCCAACGTTCTCAACAGACACCCA ATACTCCCGCTTCGGCGGGTGGGGATAACACCTGACG AAAAGGCGATGTTAGACACGCCAGGTCATAATCCCC GGAGCTTCGGCTCC	Template used for IVT
tC19Z_36_IVT	IDT, gblock	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAAAAGACAAATCTGCCCTCAGAGC TTGAGAACACAGTCGTGTGCAGAGGAGGCAGCCTTCG GTGGCGCGATAGCGCCAACGTTCTCAACAGACACCCA ATACTCCCGCTTCGGCGGGTGGGGATAACACCTGACG AAAAGGCGATGTTAGACACGCCAGGTCATAATCCCC GGAGCTTCGGCTCC	Template used for IVT
tC19Z_46_IVT	IDT, gblock	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAAAAGACAAATCTGCCCTCAGAGC TTGAGAACATCTTCGGATGCAGTCGAGGCAGCGATCG GTGGCGCGATAGCGCCAACGTTCTCAACAGACACCCA ATACTCCCGCTTCGGCGGGTGGGGATAACACCTGACG AAAAGGCGATGTTAGACACGCCAGGTCATAATCCCC GGAGCTTCGGCTCC	Template used for IVT
tC19Z_55_IVT	IDT, gblock	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAAAAGACAAATCTGCCCTCAGAGC TTGAGAACATCTTCGGATGCAGAGAAGGCAGTCTTCG GTGGCGCGATAGCGCCAACGTTCTCAACAGACACCCA ATACTCCCGCTTCGGCGGGTGGGGATAACACCTGACG AAAAGGCGATGTTAGACACGCCAGGTCATAATCCCC GGAGCTTCGGCTCC	Template used for IVT
tC19Z_73_IVT	IDT, gblock	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAAAAGACAAATCTGCCCTCAGAGC TTGAGAACATCTTCGGATGCAGAGGAGGCAGCCTTCG GTGGGACGATAGTCCCAACGTTCTCAACAGACACCCA ATACTCCCGCTTCGGCGGGTGGGGATAACACCTGACG AAAAGGCGATGTTAGACACGCCAGGTCATAATCCCC GGAGCTTCGGCTCC	Template used for IVT
tC19Z_81_IVT	IDT, gblock	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAAAAGACAAATCTGCCCTCAGAGC TTGAGAACATCTTCGGATGCAGAGGAGGCAGCCTTCT GTGGCGCGATAGCGCCATAGTTCTCAACAGACACCCA ATACTCCCGCTTCGGCGGGTGGGGATAACACCTGACG AAAAGGCGATGTTAGACACGCCAGGTCATAATCCCC GGAGCTTCGGCTCC	Template used for IVT

tC19Z_91C_IVT	IDT, gblock	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAAAAGACAAATCTGCCCTCAGAGC TTGAGAACATCTTCGGATGCAGAGGAGGCAGCCTTCG GTGGCGCGATAGCGCCAACGTTCTCAACAGTCCCCCA ATACTCCCGCTTCGGCGGGTGGGGATAACACCTGACG AAAAGGCGATGTTAGACACGCCAGGTCTAATCCCC GGAGCTTCGGCTCC	Template used for IVT
tC19Z_91G_IVT	IDT, gblock	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAAAAGACAAATCTGCCCTCAGAGC TTGAGAACATCTTCGGATGCAGAGGAGGCAGCCTTCG GTGGCGCGATAGCGCCAACGTTCTCAACAGTCCCCCA ATACTCCCGCTTCGGCGGGTGGGGATAACACCTGACG AAAAGGCGATGTTAGACACGCCAGGTCTAATCCCC GGAGCTTCGGCTCC	Template used for IVT
tC19Z_91U_IVT	IDT, gblock	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAAAAGACAAATCTGCCCTCAGAGC TTGAGAACATCTTCGGATGCAGAGGAGGCAGCCTTCG GTGGCGCGATAGCGCCAACGTTCTCAACAGTCTCCCA ATACTCCCGCTTCGGCGGGTGGGGATAACACCTGACG AAAAGGCGATGTTAGACACGCCAGGTCTAATCCCC GGAGCTTCGGCTCC	Template used for IVT
tC19Z_102_IVT	IDT, gblock	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAAAAGACAAATCTGCCCTCAGAGC TTGAGAACATCTTCGGATGCAGAGGAGGCAGCCTTCG GTGGCGCGATAGCGCCAACGTTCTCAACAGACACCCA ATAGTCCCGCTTCGGCGGGTGGGGATAACACCTGACG AAAAGGCGATGTTAGACACGCCAGGTCTAATCCCC GGAGCTTCGGCTCC	Template used for IVT
tC19Z_109_IVT	IDT, gblock	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAAAAGACAAATCTGCCCTCAGAGC TTGAGAACATCTTCGGATGCAGAGGAGGCAGCCTTCG GTGGCGCGATAGCGCCAACGTTCTCAACAGACACCCA ATACTCCACAGTCGTGTGGTGGGGATAACAGCTGACG AAAAGGCGATGTTAGACACGCCAGGTCTAATCCCC GGAGCTTCGGCTCC	Template used for IVT
tC19Z_129_IVT	IDT, gblock	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAAAAGACAAATCTGCCCTCAGAGC TTGAGAACATCTTCGGATGCAGAGGAGGCAGCCTTCG GTGGCGCGATAGCGCCAACGTTCTCAACAGACACCCA ATACTCCCGCTTCGGCGGGTGGGGATAACAGTCGACG AAAAGGCGATGTTAGACACGCCAGGTCTAATCCCC GGAGCTTCGGCTCC	Template used for IVT
tC19Z_160_IVT	IDT, gblock	GATCGATCTCGCCCGCGAAATTAATACGACTCACTAT AGTCATTGAAAAAAAAAGACAAATCTGCCCTCAGAGC TTGAGAACATCTTCGGATGCAGAGGAGGCAGCCTTCG GTGGCGCGATAGCGCCAACGTTCTCAACAGACACCCA ATACTCCCGCTTCGGCGGGTGGGGATAACAGCTGACG AAAACGCGATGTTAGACACGCGCAGTCCATAATCCCC GGAGCTTCGGCTCC	Template used for IVT

HPrz_lib_FI	IDT	ACGAATCTCTCAGGGTACCAGNNNNNTNNCAGACTT ACGTCGTGNGNNNNNCTGGTCTACNNCNNTGCCCCGA AGGGCANNNTATAGTGAGTCGTATTAATTTC	Template used for IVT of HPrz_lib
HPrz_sel_fw	IDT	CCCGCGAAATTAATACGACT	Forward primer used to recover self-ligated HPrzs by RT-PCR
HPrz_sel_rev	IDT	ACGAATCTCTCAGGGTACCAG	Reverse primer used to recover self-ligated HPrzs by RT-PCR
RPR4_fw	Sigma	GTCATTGAAAAAAAAAGACAAATCTGCC	Forward primer used for RT-PCR of full-length RPR4
RPR4_rev	IDT	GGAGCCGAAGCTCCG	Reverse primer used to for RT-PCR and gblock amplification of full-length RPR4
RPR7_fw	IDT	GTCATTGAAAAAAAAAGACAAATCTGCCCTC	Forward primer used to for RT-PCR of full-length RPR7
RPR7_rev	Sigma	GGGGATTATGACCTGGCGGTGTCTAACATCCG	Reverse primer used to for RT-PCR and gblock amplification of full-length RPR7
HPzsel_P5_21	IDT	AATGATACGGCGACCACCGAGATCTACACTCTTTCCC TACACGACGCTCTTCCGATCTNNNGTTTCGCCGCGA AATTAATACGACT	5' adapter primer for barcoding HPz selection round 1
HPzsel_P5_22	IDT	AATGATACGGCGACCACCGAGATCTACACTCTTTCCC TACACGACGCTCTTCCGATCTNNNCGTACGCCGCGA AATTAATACGACT	5' adapter primer for barcoding HPz selection round 2
HPzsel_P3	IDT	CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATCTACGAATCTCAGG GTACCAG	3' adapter primer deep-sequencing of HPrz libraries

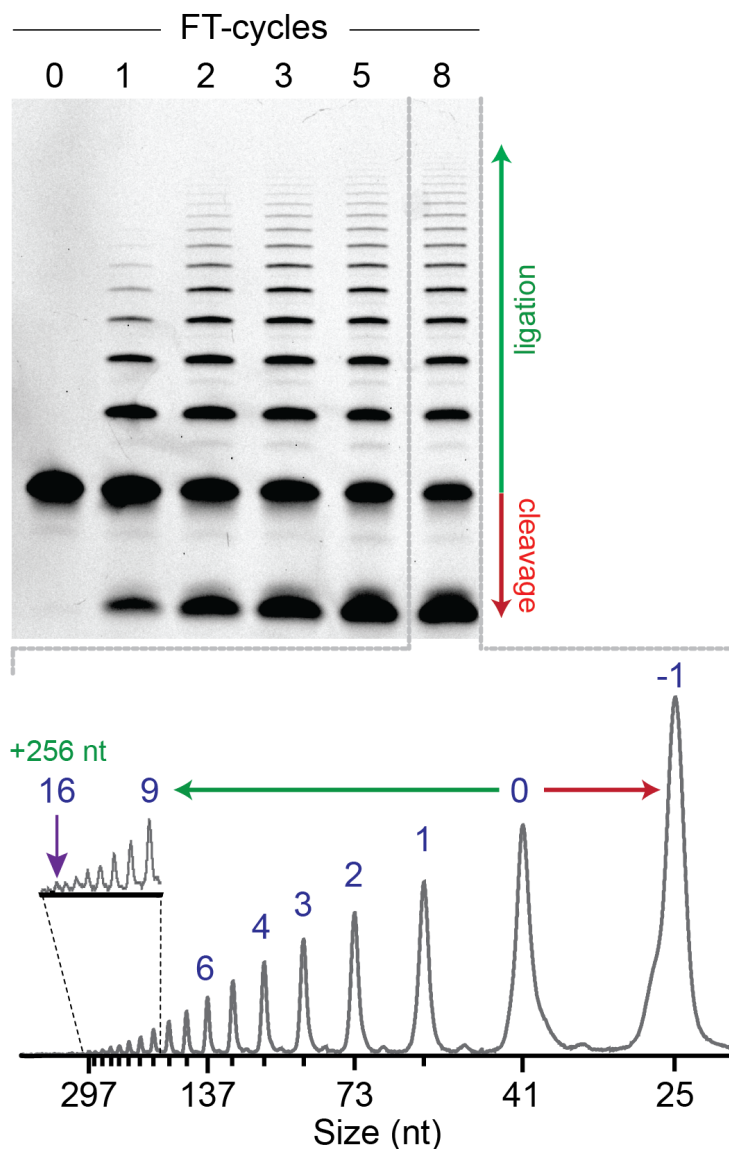


Supplementary Figure 1 | Activity of 3frHPz. **a**, 3frHPz can assemble larger RNAs from short fragments under eutectic conditions by multiple iterative ligations. During the reaction, the substrate binding strands (SBS, black) bind to specific repeat substrates (green) activated by 2', 3'-cyclic phosphates (red), which are concatenated in a reversible transesterification reaction in presence of the loop B domain (blue). The reaction (and its cleavage-ligation bias) can be visualised by an extended reporter-substrate that contains a 5'-fluorophore and an internal cleavage/ligation site. **b**, Eutectic extension and shortening of the reporter (9h, -9°C) in presence of 16-mer-repeat substrate and 3frHPz (0.3 μ M FRep16mer, 3 μ M SBS.16mer, 1 μ M loop B, 2.7 μ M 16-mer).

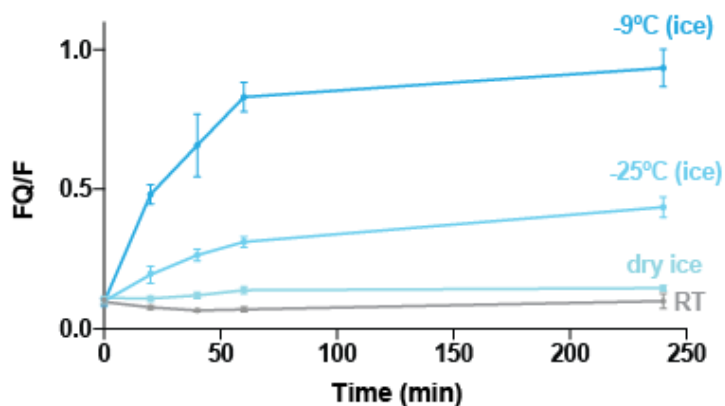


Supplementary Figure 2 | Impact on activity of introducing canonical HPz ligation sites into tC19Z.

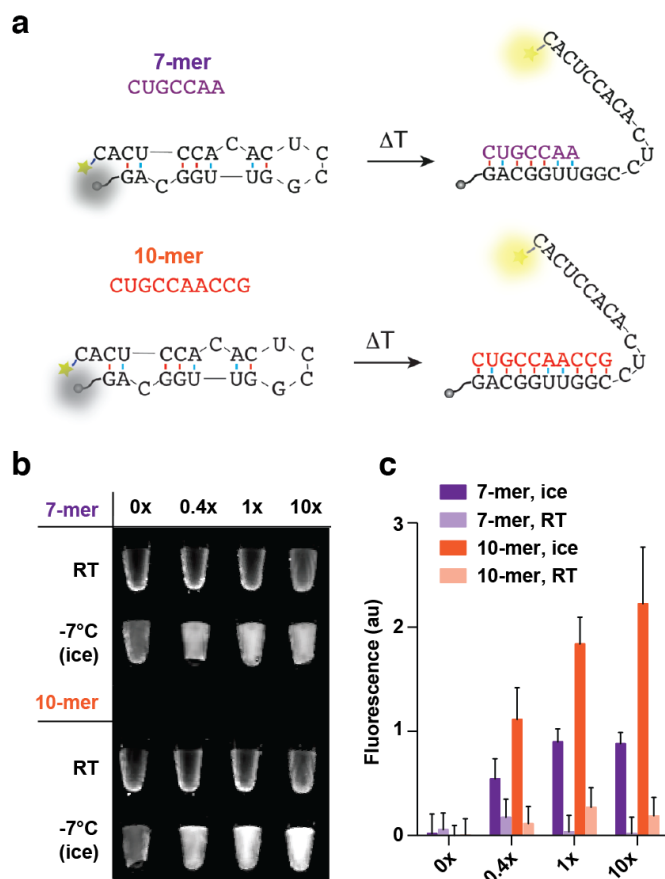
a, Sequence and predicted secondary structure of tC19Z (nucleotide numbering from the parental R18 ribozyme⁶). Colours of the ligation sites indicate the impact of the individual mutations on tC19Z primer extension activity shown in (b). Blue nucleotides indicate changes in the tC19Z sequence. **b**, Primer extension activity of the individual mutants of tC19Z (17 h, 17°C). **c**, Primer extension activity of tC19Z and RPR4 (tC19Z with mutations for the cognate HPz site 36, 91G, and 129).



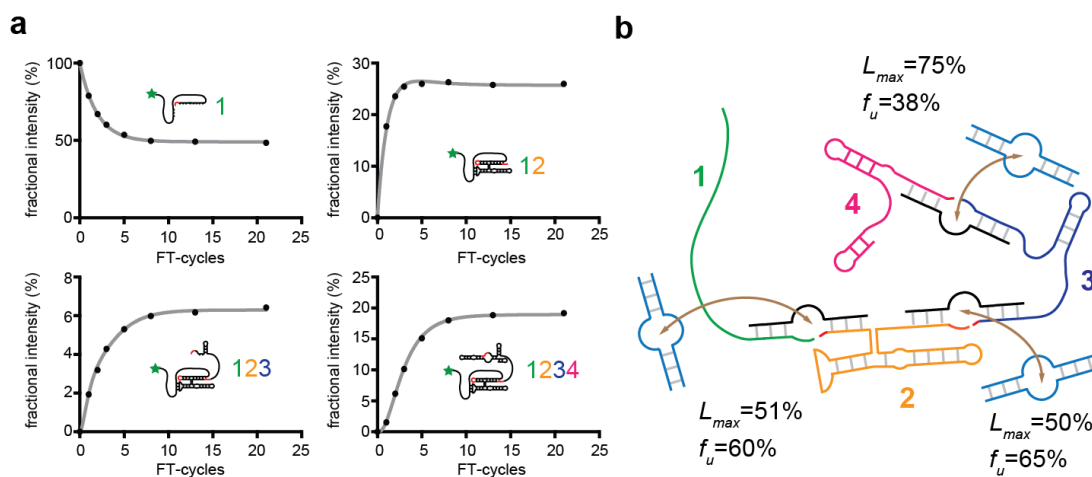
Supplementary Figure 3 | Freeze-thaw cycling boosts ligation of small oligonucleotides into large RNAs. Multiple iterative ligations of the 16-mer repeat shown in Supplementary Fig.1 and Supplementary Tab. 1 as a function of FT-cycles. A densitogram of the lane corresponding to cycle 8 (lower panel) shows that under the given conditions 3frHPz can catalyse up to 16 iterative ligations (+256 nt) creating fluorescent reporter strands with a total length of 297 nts (0.3 μ M FRep.16mer, 3 μ M SBS.16mer, 1 μ M loop B, 2.7 μ M 16mer).



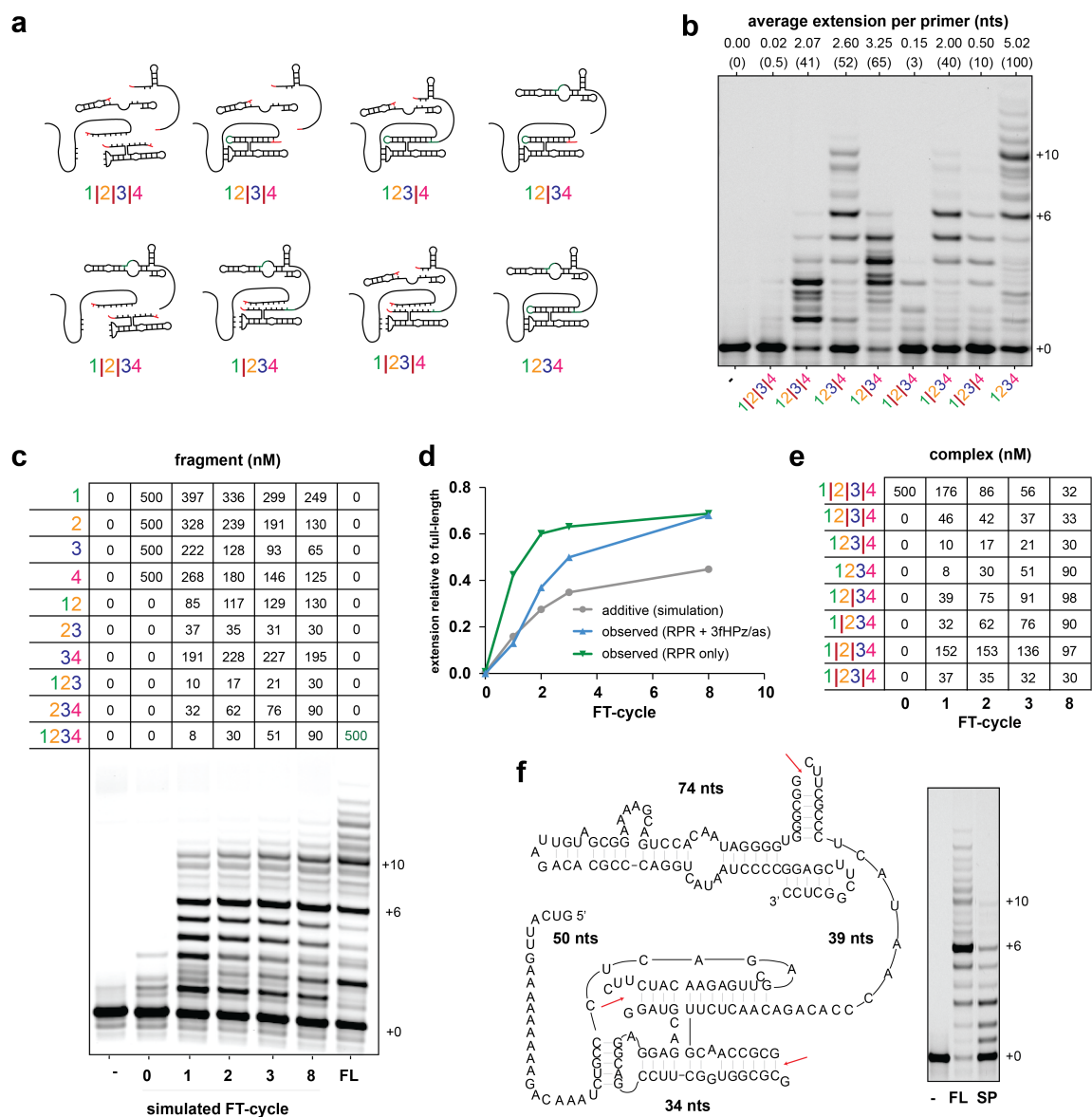
Supplementary Figure 4 | Strand invasion kinetics. The preformed F24MM_fw / 24Q_rev internal loop (0.25 μ M, Supplementary Tab. 1) was incubated at different temperatures in presence of 1 μ M 24_fw. Aliquots from a single master mix were incubated at different temperatures for the times indicated. Ice-formation in the -25°C and -9°C samples was induced by short a short freezing-step on dry ice before the start of the reaction at the respective temperatures. Triplicates of aliquots were quickly thawed in a metal block (~30 seconds at room temperature), transferred to a transparent 96-well PCR plate (StarLab) and scanned using a Typhoon Trio scanner (GE Healthcare, 3 mm focal plane, 610-nm band-pass filter). Fluorescence signals were quantified using ImageQuant TL and normalized by division by the fluorescence intensity of unquenched F24MM_fw.



Supplementary Figure 5 | Invasion of a stemloop structure by short oligonucleotides. a, Illustration of the strand invasion reaction of either a 7-mer or a 10-mer RNA oligonucleotide into a 5'-Cy5 / 3'-Iowa black RQ labelled stemloop structure **b,** Examples of the increase in Cy5 of the stemloop (62.5 nM) fluorescence in presence of increasing relative amounts (0x - 10x) of the 7- or 10-mer oligonucleotide at ambient temperature (RT) or after freezing followed by incubation at -7°C. Note that all reactions were performed in 10 mM Tris•HCl pH 7.4 and 10 mM MgCl₂. **c,** Quantification of the increase in fluorescence (n = 3) after background subtraction (0.8±0.1 au).

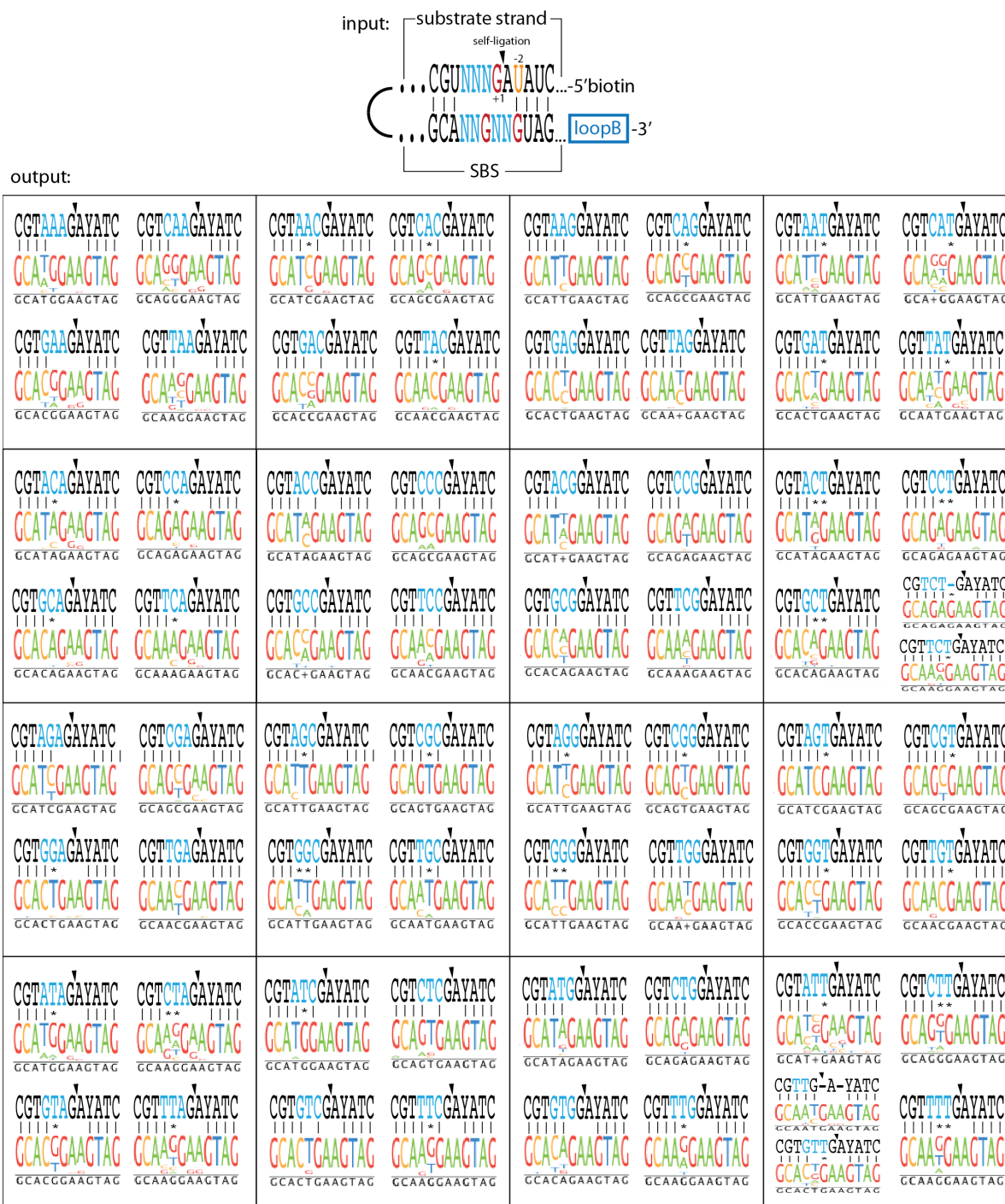


Supplementary Figure 6 | Quantification of FT-enhanced RPR4 assembly. **a**, Fractional band intensities of the four different 5' labelled RNA species that occur during assembly of RPR4 at stoichiometric fragment concentrations of 1 μM . Each point represents an average of two separate gel-quantifications. Grey lines represent the best global fit of the fractional intensities of each visible species by a model assuming independent ligation events (Supplementary equation 2). **b**, Best-fit parameters for maximal ligation yields (L_{max}) and fractions of unreacted substrate per cycle (f_u) of the individual ligation sites (stoichiometric fragment concentrations, 1 μM).



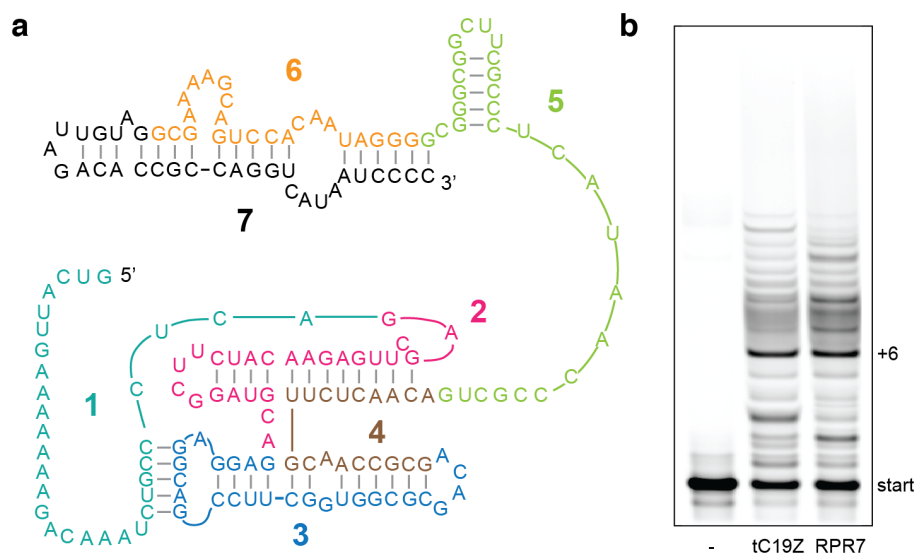
Supplementary Figure 7 | Primer extension activity of fragmented RPRs. **a**, Illustration of the eight possible RPR4 assembly intermediates. **b**, Primer extension activity of the intermediates shown in panel (a) at standard conditions (0.5 μ M of each RNA, 200 mM MgCl_2 , 4 mM NTPs, Temp1, FITC-PRIR1, 4 days at 17°C). **c**, Primer extension activity of the different PRP4 fragment / intermediate / product mixes predicted by the best fit of the original RPR4 assembly reaction (Supplementary Fig. 6) under standard conditions. Please note that the overall RPR extension activity is increased in absence of 3fHPz strands and SBS-antisense oligonucleotides. **d**, Extension activities (normalized to the full-length activity of each experiment) of reaction mixtures containing (green) the predicted fragments concentrations shown in panel (c), (blue) the assembly reaction catalysed by 3fHPz, and

(*grey*) the expected extension activity in a simplified additive scenario, which assumes that all ligation intermediates assemble into one of the non-covalent RPR4 complexes shown in panel (a) and that each of these complexes extends only a single primers according to their individual activity determined in panel b. **e**, Calculated maximal complex concentrations as predicted by the best fit (Supplementary Fig. 6) used to simulate the additive model in panel d (*grey*). **f**, Illustration and primer extension activity of a structurally guided four-fragment split of tC19Z (SP). Full-length (FL) activity is shown for reasons of comparison.

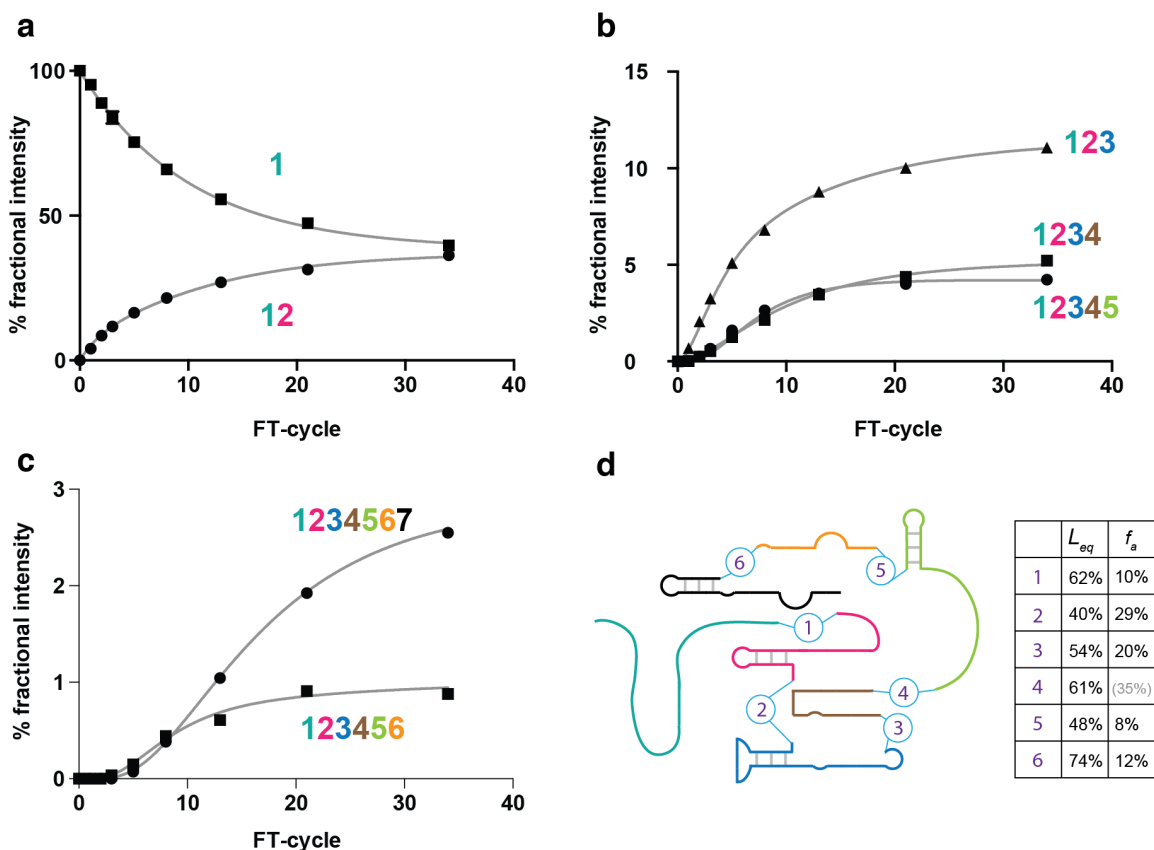


Supplementary Figure 8 | 64 different novel ligation sites (as SBS-substrate combinations) identified by *in vitro* selection. Input RNA for selection was a self-ligating HPz construct containing loop B *in cis* (top diagram and Supplementary Tab. 1). Cyan nucleotides were randomized. Red nucleotides critical for HPz activity remained unchanged. Pos -2 was U during selection to allow transcription by T7 polymerase but was changed to C in later 3frHPz ligation reactions. Black

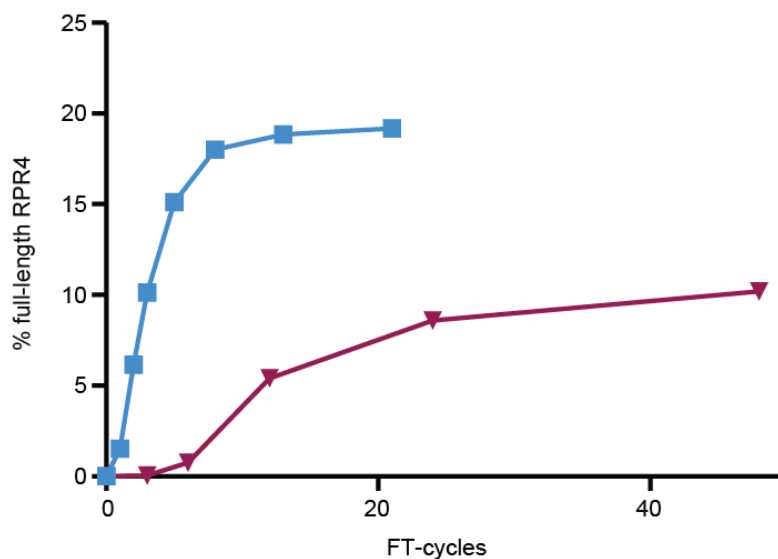
nucleotides were later adapted to their cognate RPR7 fragments. SBS consensus logos (shown as DNA) were created from the 10-30 most abundant sequences of each SBS-substrate combination after selection round 2. Some products accumulated single point deletions in the substrate strand resulting in two main SBS species for a given substrate sequence.



Supplementary Figure 9 | tC19Z variant RPR7. a, Secondary structure of RPR7. Different colours indicate fragments defined by selected HPz ligation junctions. **b**, Primer extension activity of full-length RPR7 and parental tC19Z under standard conditions (17°C, 4 d).



Supplementary Figure 10 | Quantification of FT-enhanced RPR7 assembly. **a-c**, Each panel shows fractional intensities of the different 5' labelled RNA species that occur during assembly of RPR7 shown in Fig. 5b (for concentrations see Material and methods). Each point represents an average of two independent experiments. Grey lines represent the global fit assuming independent ligation events (Material and methods), **d** Best fit parameters for the maximal ligation efficiency (L_{max}) and fraction of unreacted substrate per cycle (f_u) from the global fit shown in panel a-c. The parameter f_u for ligation site 4 did not converge and was set constant to the value obtained from the analysis of the identical ligation site in RPR4 during the final global fit.



Supplementary Figure 11 | Difference of RPR4 full-length formation when starting from >p activated fragments (blue) or inert fragments requiring tail-cleavage for activation (purple). Both experiments were performed at the same stoichiometric fragment concentrations (1 μ M) and the same amounts of 3frHPz oligonucleotides (1.1 μ M SBSs, 4 μ M loop B).

Supplemental References

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