SUPPLEMENTARY MATERIAL

Functional reconstitution and characterization of *Pyrococcus furiosus* RNase P

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Running title: Reconstitution and characterization of *Pfu* RNase P

Cloning the genes encoding the RNA and protein subunits of *Pfu* RNase P

Database mining of the *Pfu* genome confirmed the presence of an RNA and four protein subunits, which share homology to their yeast/human counterparts (Table S1).

The gene encoding *Pfu* RPR was obtained by PCR with *Pfu* genomic DNA as the template and the appropriate gene-specific primers (PfuRPR-F and PfuRPR-R; Table S2). The PCR product containing the RPR gene was digested with *Eco*RI (whose recognition site was included in PfuRPR-R, the reverse primer) and then subcloned into pBT7 (1) that had been digested with *Stu*I (which generates a blunt end) and *Eco*RI. The resulting plasmid named pBT7-*Pfu* RPR has the *Pfu* RPR gene placed under the control of a T7 RNA polymerase promoter. *Pfu* RPR can be generated using either *Eco*RI- or *Sty*I-linearized pBT7-*Pfu* RPR as template in an *in vitro* transcription reaction.

The gene encoding *Pfu* RPRΔ64-222 was obtained using a two-step cloning approach. First, the gene encoding *Pfu* RPR was amplified by PCR using primers CP-F and CP-R (Table S2) and cloned into the *Eco*RI and *Pst*I sites of pBT7-*Pfu* RPR to generate pBT7-(*Pfu* RPR)$_2$, in which two copies of the RPR gene are present in tandem. The coding sequence for *Pfu* RPRΔ64–222 was amplified by PCR using pBT7-(*Pfu* RPR)$_2$ as template and RPR 225-F and RPR 63-R as primers (Table S2). This PCR product was digested with *Bam*HI (whose recognition site was included in RPR 63-R, the reverse primer) and ligated to pBT7 that had been digested with *Stu*I (which generates a blunt end) and *Bam*HI. The resulting plasmid, which we named pBT7-*Pfu* RPRΔ64–222, when linearized with *Fok*I (whose recognition site was included in RPR 63-R, the reverse primer) could be used as the template in an *in vitro* transcription reaction to generate *Pfu* RPRΔ64-222.

The genes encoding *Pfu* Pop5, Rpp21, Rpp29 and Rpp30 were amplified by PCR with *Pfu* genomic DNA as the template and the respective gene-specific primers listed in Table S2. The PCR products were then digested with *Eco*RI, whose recognition site was included in the various reverse primers. All four protein-encoding ORFs were cloned into pET-33b that had been digested with *Nco*I, filled in with Klenow, and subsequently digested with *Eco*RI. This cloning approach ensured that no additional amino acid residues were introduced in the four ORFs.

Automated DNA sequencing at the OSU Plant-Microbe Genomics Facility was used to ascertain the sequence of the clones obtained using the procedures described above.

Generation of RPRs using *in vitro* transcription

The plasmids pBT7-*Pfu* RPR and pBT7-*Pfu* RPR Δ64-222 were linearized with *Eco*RI and *Fok I*, respectively, and used as templates for T7 RNA polymerase-mediated run-off transcription using established protocols (1, 2). The RNA transcripts thus generated were subsequently purified by dialysis and their concentrations determined from A_{260} measurements.

Protein overexpression

Escherichia coli BL21(DE3) Rosetta cells (Novagen) were transformed with pET-33b plasmids containing the gene for one of the four protein subunits of *Pfu* RNase P. Cells were grown at 37ºC in LB media containing 35 μg/ml kanamycin and 35 μg/ml chloramphenicol and induced with 2 mM isopropyl-β-D-thiogalactoside (IPTG) at OD_{600} ~0.6. For Rpp21 overexpression, 50 μM $ZnCl_2$ was also added at the time of induction as the protein was suspected to harbor a Zn^{2+}-binding motif (based on its sequence), a premise that has been borne out by recent structural studies (3, 4). All four *Pfu* Rpps overexpressed well and were purified using

cation-exchange chromatography with the exception of Rpp21, which required an additional reversed-phase chromatographic step (Fig. 1; see below).

Purification of protein subunits of *Pfu* RNase P

***Pfu* Pop5:** Cells were resuspended in denaturing buffer [25 mM Tris-HCl (pH 7.5), 7 M urea, 10 mM DTT, 0.1 mM PMSF and 1 mM EDTA], sonicated, and centrifuged for 15 min at 12,000 g. The supernatant was passed through a 0.22-μm filter and loaded on a 5-ml HiTrap SP-Sepharose column (Amersham Biosciences). Pop5 was re-folded on the column using a decreasing (7 to 0 M) urea gradient and subsequently eluted using an increasing salt gradient. Pop5 typically eluted around 1.5 M NaCl.

***Pfu* Rpp29:** Cells were resuspended in non-denaturing buffer [25 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.1 mM PMSF, and 1 mM EDTA], sonicated, and centrifuged for 15 min at 12,000 g. The pellet was then resuspended in denaturing buffer, sonicated, and centrifuged for 15 min at 12,000 g. The supernatant was filtered using a 0.22-μm filter and loaded on a 5-ml HiTrap SP-Sepharose column (Amersham Biosciences). Rpp29 was then re-folded and eluted as described above for Pop5. Rpp29 typically eluted around 1 M NaCl.

***Pfu* Rpp30:** We employed two cation-exchange chromatography steps using the same matrix (SP-Sepharose) but under successive denaturing and native conditions. We first prepared the sample in a manner similar to *Pfu* Pop5 (see above) up to the step of loading the SP-Sepharose column. Rpp30 was then eluted using an increasing salt gradient under denaturing conditions (i.e., 7 M urea). Peak fractions containing partially purified Rpp30 were pooled, dialyzed to remove salt, re-loaded on an SP-Sepharose column, re-folded using a decreasing (7 to 0 M) urea

gradient and eluted using an increasing salt gradient. Rpp30 typically eluted around 550 mM NaCl.

***Pfu* Rpp21:** Cells were resuspended in non-denaturing buffer, sonicated, and centrifuged for 15 min at 12,000 g. The pellet, containing *Pfu* Rpp21, was washed successively with the non-denaturing buffer supplemented with 3 M and 7 M urea to help solubilize proteins other than Rpp21. The final wash was centrifuged for 20 min at 14,500 g. The pellet, containing *Pfu* Rpp21, was resuspended in a stronger denaturing buffer [25 mM Tris-HCl (pH 7.5), 0.1 mM PMSF, 5 mM DTT, and 6 M guanidine hydrochloride], sonicated, and centrifuged for 20 min at 14,500 g. The supernatant was filtered using a 0.22-μm filter and applied on a C4 reversed-phase HPLC column. Elution was performed using a linear gradient of CH_3CN in 0.1% (v/v) trifluoroacetic acid. Rpp21 typically eluted at 40% (v/v) CH_3CN. The peak fractions were pooled, lyophilized, and resuspended in an acidic buffer containing 50 mM Tris-HCl (pH 2.5), 10 mM $MgCl_2$, 800 mM NH_4OAc, 1 mM DTT, 300 μM $ZnCl_2$. The pH of the solution was slowly raised to 7.5 by adding 10 M NaOH (adapted from ref. 3).

The A_{280} values for the different *Pfu* Rpps were measured and the protein concentrations calculated using their respective extinction coefficients. Purified *Pfu* Rpps were stored at room temperature and dialyzed against 50 mM Tris-HCl (pH 7.5), 30 mM $MgCl_2$ and 800 mM NH_4OAc prior to use.

Primer extension analysis to map the cleavage site in ptRNA^{Tyr}

RNase P assay was performed using *in vitro* reconstituted *Pfu* RNase P and unlabeled ptRNA^{Tyr} . The reaction contents were precipitated, washed with 70% (v/v) ethanol, air-dried and re-suspended in water. The oligonucleotide RTPR-TYR which is complementary to the 3′-end

of ptRNA^{Tyr} (Table S2) was 5′-end-labeled with γ-[^{32}P]-ATP and T4 polynucleotide kinase and used to prime the reverse transcription of mature tRNA generated by *in vitro* reconstituted or partially purified native *Pfu* RNase P holoenzymes. To minimize artificial stops caused by the secondary structure of the tRNA, the extension reactions were performed at 50°C using ThermoScript (Invitrogen) reverse transcriptase as specified by the supplier. The products of the reverse transcription reactions were separated in an 8% (w/v) polyacrylamide/7M urea gel in parallel with a DNA sequencing ladder using RTPR-TYR as the primer and ptRNA^{Tyr}-encoding DNA as the template (Fig. S2).

Partially purified native *Pfu* RNase P was obtained from a 2-g (wet weight) *Pfu* cell pellet (a kind gift from Prof. Mike Adams, University of Georgia). The cells were homogenized in extraction buffer [EB; 20 mM HEPES (pH 8.0), 5 mM DTT and 5 mM $MgCl_2$] and the cleared lysate was subjected to successive anion- and cation-exchange chromataography on DEAE- and SP-Sepharose matrices. In both cases, after extensive washing with EB, the bound proteins were eluted using a linear gradient of 0-1 M NaCl. Fractions containing RNase P were identified by their ability to process *E. coli* ptRNA^{Tyr}. The enzyme eluted at ~ 400 and 600 mM NaCl from the DEAE and SP-Sepharose matrices, respectively. The partially purified native *Pfu* RNase P holoenzyme thus obtained was free of contaminating nucleases.

Analysis of the 5′ end of the mature tRNA^{Tyr} generated by *Pfu* RNase P-mediated cleavage

RNase P assay was performed using *in vitro* reconstituted *Pfu* RNase P and ptRNA^{Tyr}, which was internally labeled with α-[^{32}P]-GTP. The reaction products were separated in an 8% (w/v) polyacrylamide/7 M urea gel and viewed by autoradiography. The band corresponding to the mature tRNA was excised from the gel. The RNA was eluted by soaking the gel slice in 350 μl

elution buffer [20 mM Tris-HCl (pH7.5), 100 mM NaCl, 0.01% (w/v) SDS and 10 mM EDTA] at room temperature for 3 h. The tRNA was precipitated by adding 2 volumes of ethanol and the pellet was resuspended in a buffer containing 50 mM sodium acetate, pH 4.5, and 20 mM EDTA and digested with 0.05 U RNase T2 (Invitrogen) for 5 h at 37°C. The hydrolytic products (from RNase T2 digestion) were separated by polyethyleneimine-cellulose thin layer chromatography using 1 M ammonium formate (pH 3.5) as the solvent and viewed by autoradiography (Fig. S3, ref. 5).

Optimization of assay conditions for *Pfu* RPR in the absence and presence of its Rpps

Protein titration: To obtain near-complete assembly of the RNA subunit to form the different RNP complexes, it is important to have some knowledge of the K_D values for the binding of the protein subunits to the RNA moiety. We used a gel-shift assay to assess the ability of each of the four *Pfu* Rpps to bind *Pfu* RPR either individually or as binary complexes. As expected from activity assays where no single protein subunit could confer even weak activity on the RNA (Fig. 2), we failed to detect specific binding between individual protein subunits and the RNA moiety (data not shown). Although we could detect some mobility-retarded species consistent with the presence of RNA bound to Rpp pairs, non-specific large aggregates prevented us from obtaining reliable K_D values. In the absence of this information, we had to rely on protein titration experiments to determine the protein:RNA ratios that would permit the maximum RNP complex formation for the various *Pfu* RPR and Rpp combinations. We titrated different amounts of the protein subunits to a constant amount of *Pfu* RPR and assayed for activity under optimal Mg^{2+} concentrations (Table 3). The concentration of *Pfu* RPR had to be varied for the different reconstitution assays to keep the assay incubation time within reasonable limits. Our results

indicated that (i) 500 nM *Pfu* RPR + 1.25 μM each of Rpp21 and Rpp29, (ii) 50 nM + 500 nM each of Rpp30 and Pop5, and (iii) 10 nM *Pfu* RPR + 100 nM each of all four Rpps, were optimal for the respective reconstitutions. Due to reasons unclear at the current time, Rpp concentrations >125 nM result in decreased activity in the four-protein reconstitution assay.

Monovalent and divalent cations: By varying the NH_4OAc concentrations in the assay while keeping the $[Mg^{2+}]$ constant, we determined that the *Pfu* RPR, *Pfu* RPR+ 2 Rpps, and *Pfu* RPR + 4 Rpps function most efficiently at 2 M, 100 mM and 800 mM NH_4^+, respectively. The 800 mM requirement of the fully reconstituted *Pfu* RNase P (i.e., RPR + 4 Rpps) is consistent with the 700-900 mM intracellular concentration of K^+ in *Pfu* (6). By varying the $MgCl_2$ concentrations in the assay while keeping the $[NH_4^+]$ constant, we established that the *Pfu* RPR, *Pfu* RPR+ 2 Rpps, and *Pfu* RPR + 4 Rpps function most efficiently at 500 mM, 120 mM and 30 mM Mg^{2+}, respectively.

While varying the concentration of either the monovalent or divalent cation, the concentration chosen for the invariable cation in the initial trials obviously did not coincide with the optima subsequently determined. Therefore, once the optima for $[NH_4^+]$ and $[Mg^{2+}]$ were determined, we repeated some of the analyses with the invariable cation being fixed at its optimal concentration and reconfirmed the optimal concentration for the variable cation. Moreover, we also confirmed that using different RPR:Rpp ratios (while assembling the fully reconstituted *Pfu* RNase P) did not alter the optima for $[NH_4^+]$ and $[Mg^{2+}]$.

Assay temperature: By changing the assay temperature from 38 to 88ºC, we determined that *in vitro* reconstituted *Pfu* RNase P (i.e., RPR and four Rpps) cleaves $ptRNA^{Tyr}$ optimally at 55ºC. Therefore, this temperature was chosen for all assays.

Kinetic studies

To obtain initial velocities that could then be used in Michaelis-Menten analyses, we performed assays under optimal conditions for either *Pfu* RPR alone or various partially and fully reconstituted *Pfu* RNase P holoenzymes. While all reactions were performed in 50 mM Tris-HCl (pH 7.5), varying amounts of salt were used based on the catalytic entity being tested (Table S3). The substrate concentration range used was different for each case and was chosen based on initial trials to determine the approximate K_m values (Table S3). All reactions were initiated with a pre-incubation for 5 min at 37°C followed by 10 min at 55°C. After a defined time of incubation at 55°C, the reactions were quenched with urea-phenol dye [8 M urea, 0.04% (w/v) bromophenol blue, 0.04% (w/v) xylene cyanol, 0.8 mM EDTA, 20% (v/v) phenol].

Some other comments warrant mention. For the *Pfu* RPR reactions, we chose to use 500 mM Mg^{2+} instead of 600 mM where the activity peaks (Fig. S4), since (i) we observed nearly 95% of the activity at 500 mM, and (ii) RNA precipitation could be minimized by using this lower concentration of Mg^{2+}. Although we sought to use a substrate concentration range that encompassed at least a few fold above and below each K_m value, this was not possible in two cases due to technical problems. First, for the RPR alone reaction, concentrations higher than 60 μM result in precipitation. Second, with the partially reconstituted *Pfu* RNase P made up of *Pfu* RPR+Rpp21+Rpp29, inhibition of activity was observed at substrate concentrations exceeding 14 μM. Therefore, in these two cases, the highest substrate concentration tested was only twice the estimated K_m value.

Based on phosphorimager-based quantitation, the extent of reaction (in the linear range) in each assay was calculated. These data were then subjected to linear regressions analyses on Excel to derive the initial velocities. The Michaelis-Menten curves were generated using the

curve-fitting option in Kaleidagraph. The errors for both K_m and V_{max} were ≤ 25%. The V_{max} values were used to calculate the respective k_{cat} values based on the assumption that all of the RPR in the assay does assemble into the respective partial or complete holoenzyme.

Use of RNase T1-based footprinting to map RNA-protein interactions in *Pfu* RNase P

Each 20-μl footprinting reaction contained a mix of 5′end-labeled (100,000 dpm, ~500 pM) and unlabeled (500 nM) RPRΔ64-222 either alone or complexed with Rpp21+Rpp29 or Pop5+Rpp30 in 50 mM Tris-acetate (pH 7.5), 120 mM $Mg(OAc)_2$, 400 mM $NH_4(OAc)$. The reaction mix was pre-incubated for 10 min at 37 °C followed by 10 min at 55°C. RNase T1 (0.002 U, Ambion) was added to the reconstitution mix and the incubation was continued at 55°C for an additional 4 min. The reaction was terminated by adding 10 μl of buffer -saturated phenol (pH 8) followed by extraction with phenol/chloroform. The RNA was precipitated by adding 2 volumes of ethanol in the presence of 0.3 M sodium acetate (pH 5.2) and 20 μg/ml glycogen. The RNA sample was pelleted at 18,000 g for 15 min in a micro-centrifuge and the pellet was washed twice with 70% (v/v) ethanol. The air-dried RNA sample was resuspended in 10 μl loading dye [9 M urea, 0.9 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol, 10% (v/v) phenol] and separated by 8% (w/v) polyacrylamide/7M urea gel electrophoresis. The bands were visualized by using a phosphorimager (Molecular Dynamics).

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Table S1. Characteristics of the protein subunits of *Pfu* RNase P

Rpp	Gene ID	Isoelectric point (pI)	Predicted mass, Da	Observed mass, Da*
Pop5	PF1378	10.0	13,708	13,708
Rpp21	PF1613	10.3	14,172	14,170
Rpp29	PF1816	10.2	14,953	14,954
Rpp30	PF1914	9.7	24,363	24,365

* The actual molecular masses of all four proteins were measured by electrospray ionization mass spectrometry. With the exception of Rpp29, the N-terminal Met is removed from the three other *Pfu* Rpps during overexpression in *E. coli.* Therefore, the predicted masses are calculated for the N-terminal Met-lacking variants in the case of Pop5, Rpp21 and Rpp30.

Table S2. Oligonucleotides used in this study

PfuRPR-F	5'-TAGGCGAGGGGGCTG-3'
PfuRPR-R	5'-GC*GAATT*CCTAGGCGAGGGGGCTATAG-3'
PfuPOP5-F	5'-AGTGAGCGTCCAAAAACCTTACCTCCTAC-3'
PfuPOP5-R	5'-GC*GAATTC*AACGCCACCCGAATTGAGACAG-3'
PfuRPP21-F	5'-GCTAAATACAATGAGAAAAAAGAAAAAAAGAGAATTG-3'
PfuRPP21-R	5'-GC*GAATTC*AATATTCCATTTTTTCTTTTCTTCTC-3'
PfuRPP29-F	5'-TGGCGTAACAGCGAAGAACGTGAGAATAG-3'
PfuRPP29-R	5'-GC*GAATTC*ATTTACGCCAACGCTTCTTCAGACGCATCTC-3'
PfuRPP30-F	5'-GCTGGTGGTCGTAATGGTGTGAAGTTTGTAGAGATG-3'
PfuRPP30-R	5'-GC*GAATTC*ATTTAAGACGTTCCAGAAT**a**CCAAGTGGATAAAAG-3'
CP-F	5'-*AAACTGCAG*TAGGCGAGGGGGCTGGGGGCTGGG-3'
CP-R	5'-*GGGGATCC*TAGGCGAGGGGGCTATAGCCCGC-3'
RPR 225-F	5'-TGCAAGGCCGAGTTAGGGCCGATG-3'
RPR 63-R	5'-GG*GGATCC*GGATGGACGTCTCGCCCCGGTGCGGTGGGCGGAAG-3'
RTPR-TYR	5'-GGTGGGGGAAGGATTCG-3'

*Note***:** The italicized and underlined sequences indicate the restriction sites introduced for cloning and linearization of template (for *in vitro* transcription), respectively. The nucleotide in bold, lowercase font (in PfuRpp30-R) refers to a silent substitution that eliminates an internal *Eco*RI site. All oligonucleotides were purchased from either Integrated DNA Technologies or Sigma Genosys.

Table S3. Assay conditions used for determining the steady-state kinetic parameters

Combination tested	$[NH_4^+]$, mM	$[Mg^{2+}]$, mM	[RPR]:[Protein], nM	[Substrate], mM
RPR alone	2000	500	500:0	1.4 - 56
RPR+Rpp21+Rpp29	100	120	500:1250	0.7 - 14
RPR+Pop5+Rpp30	100	120	50:500	0.7 - 50
RPR+Rpp21+Rpp29+ Pop5+Rpp30	800	30	10:100	0.025 - 2

Legends to supplementary figures

Figure S1. *Pfu* RPR-mediated cleavage of ptRNA^{Tyr}. (A) A typical time-course observed during cleavage of ptRNA^{Tyr} by *Pfu* RPR. The assay was performed in 50 mM Tris-HCl (pH 7.5), 500 mM $MgCl_2$ and 2 M NH_4OAc at 55ºC using 500 nM of *Pfu* RPR and 30.5 μM of ptRNA^{Tyr}. Note that due to the high concentration of Mg^{2+} and the high temperature used in the assay, ptRNA^{Tyr} in the absence of any catalyst undergoes modest degradation (control lanes; arrows on the left); however, these products migrate at different positions compared to the RPR-mediated cleavage products (*Pfu* RPR lanes; arrowheads on the right). (B) The initial velocity for the reaction in panel A was calculated using phosphorimager analysis and used to determine the turnover number (0.2 min^{-1}).

Figure S2. Primer-extension analysis. (A) Secondary structure representation of ptRNA^{Tyr} substrate used in the RNase P assays. (B) Lanes GATC represent the sequencing ladder generated with sthe plasmid used to transcribe ptRNA^{Tyr} and aid in locating the terminating residues of the RT reactions. Lane S represents cDNA generated from unprocessed tRNA^{Tyr} substrate. N and R depict the cDNAs generated from reactions in which ptRNA^{Tyr} was partially processed by native and reconstituted *Pfu* RNase P, respectively.

Figure S3. End-group analysis of mature tRNA generated by *E. coli* or *Pfu* RNase P-mediated cleavage. RNase P assays were performed using as substrate ptRNA^{Tyr}, labeled with α-[^{32}P]-GTP. Mature tRNA thus generated was recovered (after gel electrophoresis) and subjected to RNase T2 digestion. The digestion products were separated by TLC and identified by autoradiography. Lane 1, unprocessed ptRNA^{Tyr} (negative control); lane 2, reaction catalyzed

by *E. coli* RNase P (positive control); lane 3; *Pfu* RPR+Rpp21+Rpp29; lane 4, *Pfu* RPR + Pop5+Rpp30; lane 5, *Pfu* RPR+Rpp21+Rpp29+Pop5+Rpp30; and lane 6, *Pfu* RPRΔ64-222+Pop5+Rpp30. O indicates the origin, where the samples were spotted.

Figure S4. Determination of Mg^{2+} concentrations required for maximal activity of partially and fully assembled *Pfu* RNase P holoenzymes. The Y-axis represents relative activity where 100% represents the maximum activity observed in each case. Mean values of initial velocities were calculated using data from at least two independent experiments.

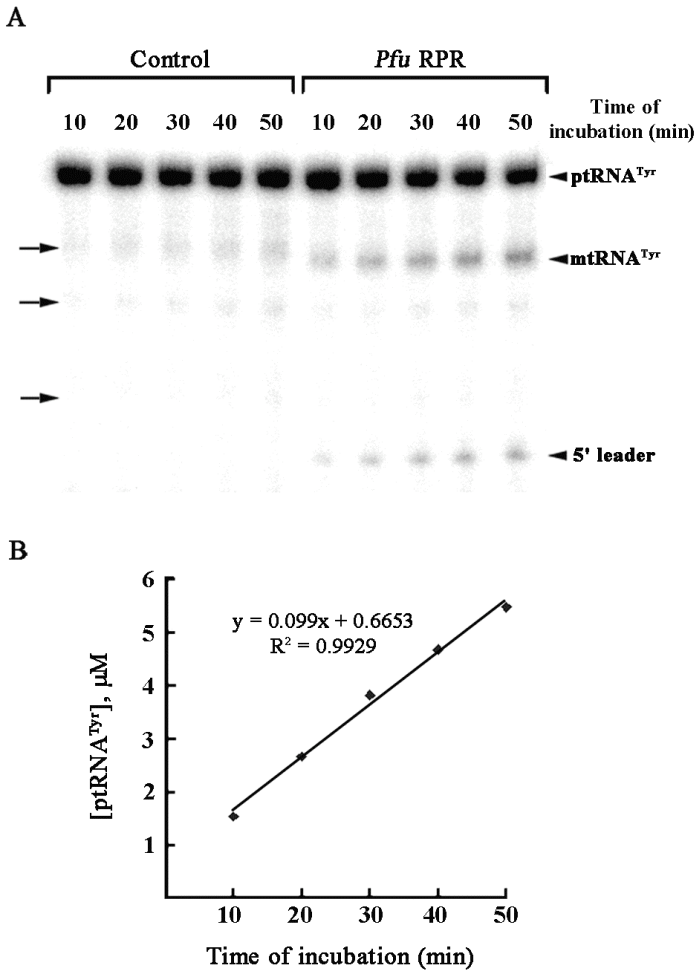
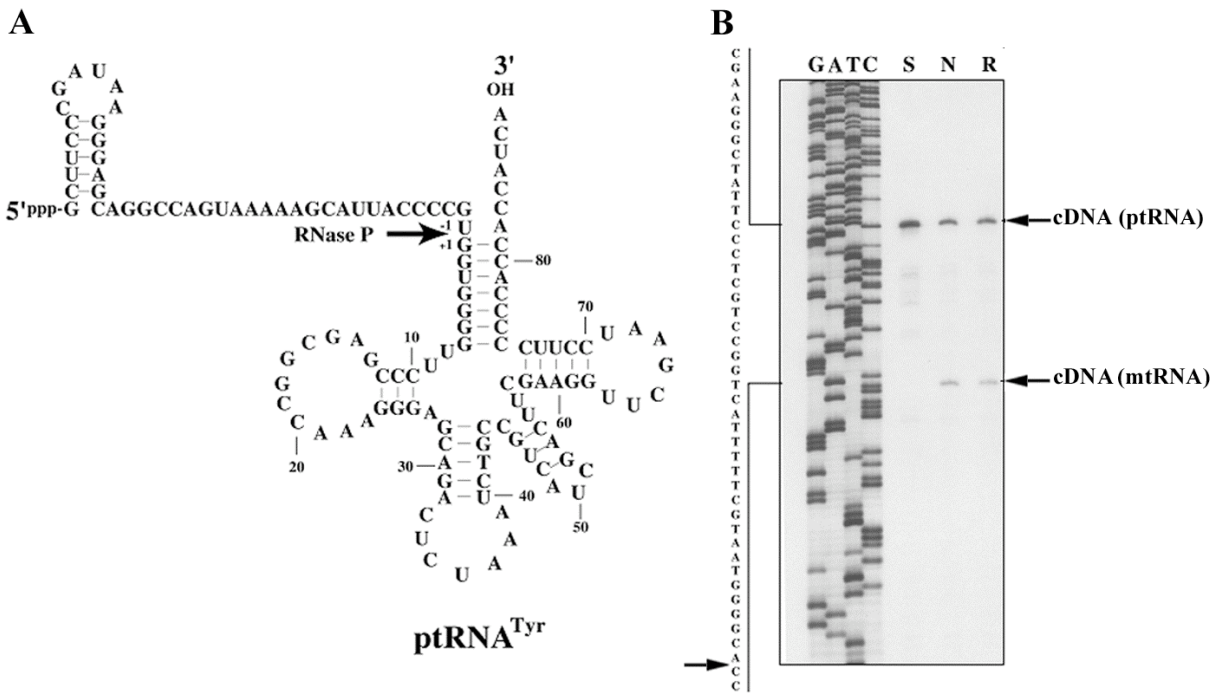

A
Control
Pfu RPR
10 20 30 40 50 10 20 30 40 50
Time of incubation (min)
ptRNA^Tyr
mtRNA^Tyr
5' leader
B
y = 0.099x + 0.6653
R² = 0.9929
[ptRNA^Tyr], μM
1 2 3 4 5 6
10 20 30 40 50
Time of incubation (min)

Figure S1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B
5'ppp-G CAGGCCAGUAAAAAGCAUUACCCCG
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G A T C
S
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cDNA (ptRNA)
cDNA (mtRNA)

Figure S2

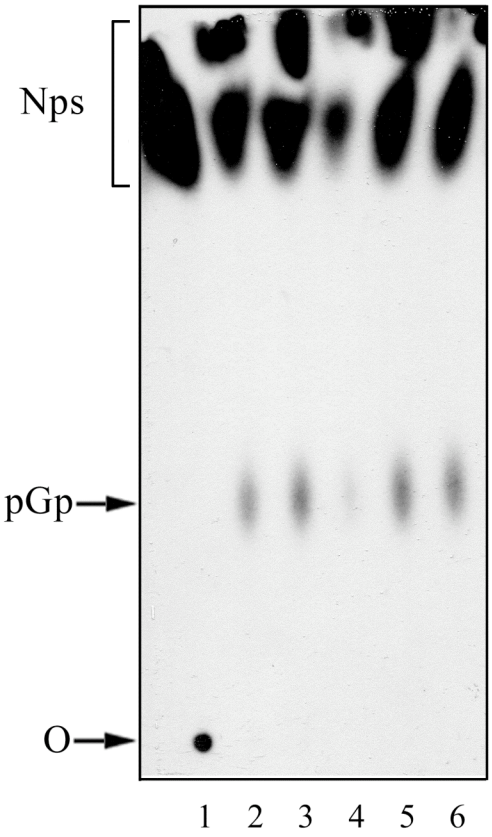

Figure S3