

**DISSECTING THE KINETICS OF RNASE E-DEPENDENT RNA  
DEGRADATION**

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## Abstract

RNase E, a bacterial endonuclease, catalyzes the cleavage of several different species of RNA. Previously it has been shown that RNase E preferentially cleaves 2 nucleotides downstream of a uracil residue. Currently it is unknown how RNase E recognizes an upstream uracil. Here, we develop an in vitro system whereby the kinetics of RNase E-dependent cleavage of small RNAs can be studied to investigate the uracil recognition mechanism of RNase E. This in vitro system comprises the construction of the catalytic domain of this RNase E (RNase E D529) and production of two fluorescently labelled small RNAs (RprA and ArcZ). This system can then be used to validate experimentally the cleavage mechanism of RNase E proposed by Chao, et all [3] whereby recognition of the upstream uracil causes a conformational change in the backbone of the RNA which facilitates cleavage.

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## Introduction

**RNase E.** RNase E is an essential bacterial endonucleases with roles in the degradation of mRNAs and the processing of different RNA species such as tRNA, rRNA and sRNAs [1, 9]. RNase E forms a core of a multienzyme assembly called the RNA degradosome [6]. RNase E encoding gene (Rne) has 1061 amino acids residues where its N-terminal domain contains the catalytic domain (1-529 residues) while the C-terminal domain (530-1061) contains specific binding sites for various proteins including polynucleotide phosphorylase (PNPase), enolase, and the helicase RhlB. In vivo RNase E exists as a tetramer composed of a dimer of dimers [2]. However, it has been shown that each dimer of RNase E is capable of cleaving and processing RNA [4]. The catalytic domain of RNase E has been shown to be capable of cleaving and processing RNA in the absence of the C-terminal domain, and is utilized to study the enzyme *in vitro* as it does not require the other components of the degradasome to be stable [8].

**RprA and ArcZ.** RprA and ArcZ are small non-coding RNAs recruited by Hfq to mRNA to perform post-transcriptional gene regulation [5, 7]. They are being used as to study the mechanism of RNase E as they have been shown to be processed to shorter stable species by the enzyme [3]. In Chao *et al.*, they performed *in vitro* experiments to show that both RNA are cleaved by RNase E and that this cleavage is dependent on the upstream uracil. Upon mutation of the uracil the RNA molecules were no longer processed. Therefore, we are using these two RNAs as a model system to study the kinetics and mechanism

of the upstream uracil dependent mechanism of RNase E RNA cleavage.

**Cleavage mechanism from recent simulations.** Previously Based on simulations [3], RNase E catalyzes the cleavage. RNase E causes distortion in the RNA backbone that opens up these bonds and expands the angle of these two bonds and allow the water molecule to access to phosphate molecule to attach. Then, nucleophilic substitution happens leading to cleavage of the RNA. This distortion is necessary for recognition of the cleavage site. In addition unpublished simulation results have suggested that RNase E may scan along an RNA molecule until it recognizes the correct upstream uracil for cleavage. Once RNase E recognizes the upstream uracil the protein will stall on this site allowing for the protein to perform RNA cleavage.

**Objectives.** In the project, we are going to develop an *in vitro* system that can be utilized to perform a stop flow experiments to study the rates of cleavage for RNA and to get a better understanding the upstream uracil dependent cleavage mechanism and potential scanning mechanism. We are going to use a FRET based assay with the FRET pair of tryptophan (Donor) and pyrene (acceptor). Therefore, we will produce pyrene-labelled RprA and ArcZ molecules, which will be rapidly mixed with purified RNase E  $\Delta$ 529 in the stopped flow. When the pyrene labelled RNA and RNase E approach each other, the system should adopt a high FRET state that can be observed. If the scanning mechanism of RNase E along the RNA is occurring, we expect an increase in FRET efficiency that will increase at intervals corresponding to RNase E scanning on the RNA. In addition we can observe when RNase E recognizes its target and dissociates from changes in FRET efficiency. Finally using non-cognate sequences of both RprA and ArcZ we can understand how the upstream uracil alters the rates of RNA cleavage.

## **Material and methods**

### **◇◇ Preparation of RNase E Δ529**

**Construction of plasmid encoding gene for Δ529 RNase E:** A Pet21b plasmid which encodes for the entire RNase E protein was received from Eric Masses lab in Sherbrook. To construct the pet21b:Δ529 rne plasmid, a Polymerase Chain Reaction (PCR) was performed using a forward and reverse primer (see Appendix). The PCR was performed with 0.4 mM dNTP, in Pfu Buffer, with 50ng of pet21b:rne and 3 units of Pfu Polymerase. The PCR was performed at three different annealing temperatures (61°C, 63.5°C, 65°C). The PCR product was treated with DpnI in Buffer Tango and incubated overnight at 37C. The PCR products were 5-phosphorylated by combining T4 PNK, ATP, DNA, 10× reaction buffer A which were thoroughly mixed and incubated at 37° for 20 minutes. The reaction was then heated it at 75° for 10 minutes to kill DpnI enzyme. A ligation reaction was then performed where we added 10× Ligation buffer, T4 Ligase and water to tube and mixed it thoroughly and incubated at room temperature overnight. Plasmid concentration was quantified by Bio-drop spectrophotometer.

**Transformation into DH5α:** Two methods for transforming plasmids into either DH5a or BL21 (DE3) strains of E. coli were used: Standard heat-shock transformation into chemically competent cells and electroporation into electro-competent cells. Here both methods worked for transforming the plasmid into cells.

**Sequencing:** Plasmids from DH5a cells were extracted using the miniprep kit (biobasic).

These plasmids were sent for sequencing at genewiz.

**Overexpression of Δ529 RNase E:** 200ml of LB Media was inoculated with BL21 DE3 cells containing D529 RNase E (including FLAG-tag) and 200 $\mu$ L Ampicilin over night at 37C shaking at 200rpm. The culture was centrifuged at 5000g for 7 min and the supernatant was removed and the pellet was re-suspended in a 1 ml fresh media (a mixture). Afterwards, 2L of LB media containing 2mL Ampicilin was inoculated to an OD<sub>600</sub> of 0 : 1 using the re-suspended cell pellet. The OD<sub>600</sub> of the flasks was measured until the OD<sub>600</sub> reached 0.6, where IPTG was added and a 1 OD<sub>600</sub> samples was taken every hour for 3 hours.

## ◇◇ Preparation of ArcZ and RprA

**Oligonucleotide assembly of arcZ and rprA gene:** DNA encoding for ArcZ and RprA were constructed using oligonucleotide assembly with four short-stranded oligonucleotides (purchased from IDT). To make 100 $\mu$ L of each of the gene (rprA and arcZ), we mixed 5 $\mu$ L of each 5M primers with 2 $\mu$ L 10mM DNTPs, 20 $\mu$ L of 5 Phusion HF buffer, 1 $\mu$ L of Phusion DNA polymerase and 57 $\mu$ L of miliQ dH<sub>2</sub>O. The oligonucleotides were assembled with annealing temperatures of 62°C for arcZ and 70°C for rprA.

**Gel extraction of DNA product and ligation into pJet:** Products from the oligonucleotide assembly were gel extracted . To this end a native polyacrylamide gel in TBE was ran 40 min at 60V, the bands corresponding to the correct length of each gene were gel extracted and placed into a microcentrifuge tube where they were crushed it with a pipette tip. The gel fragments were soaked in after soaking overnight while shaking at 22°C, the gel was centrifuged at max speed for 10 minutes. The aqueous phase was extracted and equal amounts of chloroform was added, vortexed for 30s, and centrifuged again at max speed for 2 minutes. This was repeated three times. To the final aqueous phase 1 : 10 volume of 3M Na Acetate, 3 volume of 98% ethanol and 0 : 5% LPA was added and incubated it for 1 hour at 80°C. The sample was centrifuged at 4°C for 30 min at max speed. Finally, the supernatant removed and 10 $\mu$ L

of miliQ dH<sub>2</sub>O was added to tube.

Then, we performed cloneJET ligation reaction. We combined the DNA sample with DNA Blunting enzyme and 2× reaction buffer and water, then vortexed it for 5 seconds and incubated the reaction mixture at 70°C for 5 minutes. Next, we added pJET 1.2/Blunt cloning vector (50ng/μL) and T4 Ligase into mixture and incubated it at room temperature for 5 minutes.

**Amplification by PCR:** In this step, we did PCR in which we used two primers (see Appendix) to get DNA templates of rprA and arcZ genes.

**In vitro transcription:** The following components were combined at room temperature: TraB (transcription buffer), 100mM DTT, 25mM NTPs, 100mM GMP, 0,5 U/μ L iPPase, T7 RNA polymerase, 40 U/μ L RNase Inhibitor and the template DNA to a final volume of 1ml. Then the reaction mixture was incubated at 37 C overnight. After incubation 2U/ml (2μ L) of DNase I (1U/μ L, Fermentas) was added to the in vitro transcription product and incubated for 1h at 37°C.

**Purification of RNAs:** We purified both rprA and arcZ using superdex 75 column. We started purification of ArcZ and RprA by degassing the buffer (water). Then we equilibrated AKTA prime system with 60 ml of H<sub>2</sub>O and then equilibrated 24ml Superdex 75 column with 3 columns of water and rinsed sample loop with 3 volumes of water. Afterwards, we loaded RNAs in LOAD position and switched it to INJECT position. The flow rate was 0.3ml/min and the fraction size was 0.5ml. Then we ran sample for 70ml. At the end, we collected samples and ran a urea gel on them to see which contain RNAs.

**Labelling of RNAs:** ArcZ and RprA was oxidized by incubating each RNA with 10mM Potassium periodate and 3M Na Acetate with miliQ dH<sub>2</sub>O to the final volume of 200L and incubated it on ice for 30 minutes. the reaction was stopped by adding ethylene glycol (stock 200 mM) to a final concentration of 10mM and incubated it on ice for 10 minutes. Next, the RNA was precipitated by adding 1 : 10 Volumes of 3M Na acetate and 3times volume of 99% ethanol and incubated 2 hours at -20°C. The sample was centrifuged at

## 2. MATERIAL AND METHODS

4 C for 30 minutes and at max speed and then the supernatant was removed. The pellet was incubated with pyrene and Na acetate and miliQ dH<sub>2</sub>O to a final volume of 200 $\mu$ L and incubated it on ice overnight in dark. The reaction was stopped by adding Na acetate and water and did phenol extraction process followed by two ethanol precipitation and then incubated at 4°C for 2 hours. Then, we centrifuged the sample for 30 minutes at max speed and removed supernatant. Finally, the pellet was re-suspended in 20 $\mu$ L miliQ dH<sub>2</sub>O.

**Fluorescence spectroscopy:** To measure the excitation and emission wavelengths of the labbeled RNAs, we used QuantaMaster fluorescence spectroscopy(Photon Technology International, London, ON, Canada) with a 3 mm x 3 mm quartz cuvette (Starna Cells, Inc., Atascadero, CA, USA).

## Results

**The plasmid encoding RNase E Δ529 was constructed.** Fig. 3.1 shows a native gel where there is the band at a size consistant with the construction of a pet21b: Δ529rne plasmid (7029bp). Because there is also another band with low molecular weight around 2500bp, we performed the gel extraction. Afterwards, to improve the transformation of this constructed plasmid it was phosphorylated and ligated. Then we transformed the plasmid

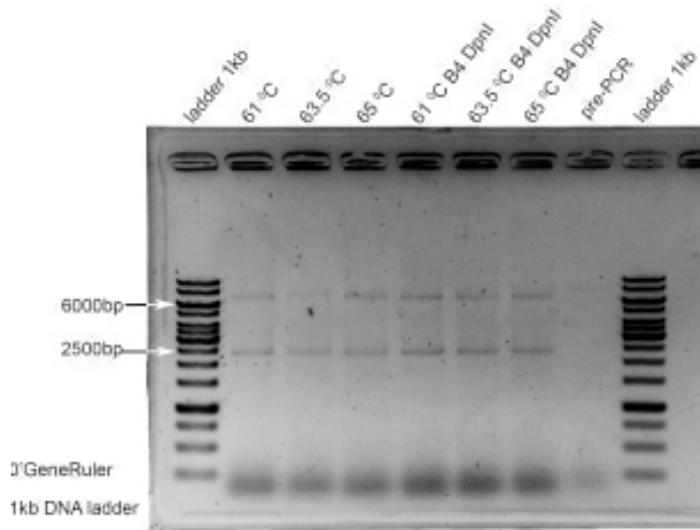


Figure 3.1: Agorose gel electrophoresis run under 100 V for 35 minutes shows the PCR product containing truncated RNase E (7029bp) plus flag tag at different annealing temperatures before and after DpnI digestion.

successfully into DH5 $\alpha$  cells and restored them. The day after we minipreped the plasmid out of the DH5 $\alpha$  cells and sent them for sequencing. Upon sequencing confirmation, the pet21b: Δ529rne plasmid was transformed into BL21 (DE3) Gold cells. Both the DH5 and

BL21 (DE3) Gold cells containing the pet21b:  $\Delta$ 529rne plasmid were stored at -80°C.

**Overexpression of RNase E  $\Delta$  529 containing flag-tag:** Fig 3.2 left shows the cell culture growth curve which corresponds to a normal E. coli growth curve with the lag, exponential, and stationary phase. However, the total optical density of the cells when they reached stationary phase is lower (~ 1.8) than what is normal for E. coli cells (~ 3.0). Fig 3.2 right shows RNase E  $\Delta$ 529-Flag (60.6 kDa) overexpression. However, it is a low yield for an overexpression. It might be because of the fact that RNase E is not healthy to overexpress in the cell.

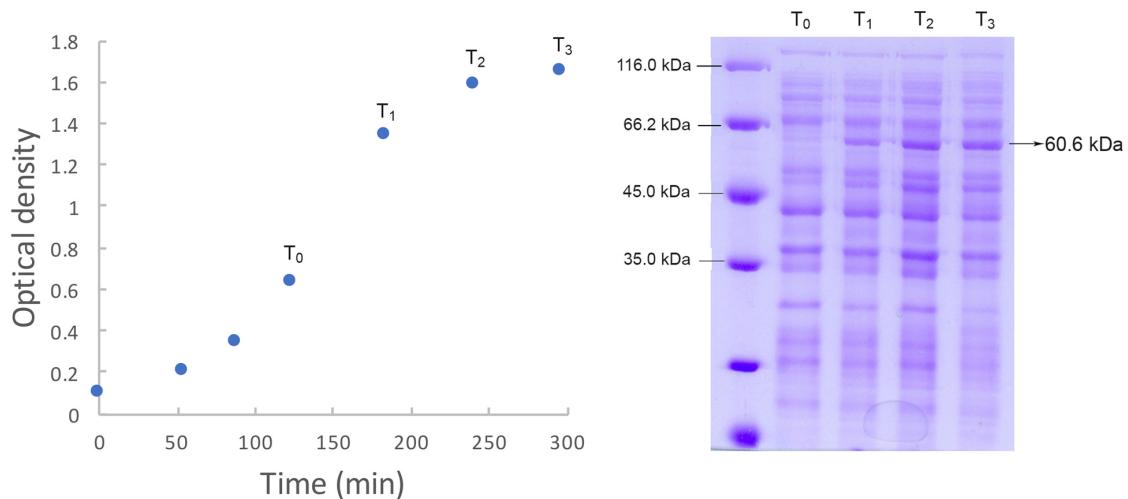


Figure 3.2: The overexpression of RNase E  $\Delta$ 529. (left) Expression culture growth. (right) SDS-PAGE showing the overexpression of RNase E at different time steps.

**Adding HIS-tag to C-terminus of the RNase E  $\Delta$  529:** To obtain a better yield in purification of our protein, We added His-tag on the C-terminus of the protein where a Flag-tag already existed using a similar technique as constructing pet21b: $\Delta$ 529rne. As shown in the native gel in Fig. 3.3 a, there is a single band corresponding to the size of the constructed plasmid in all tested annealing temperatures. Then, it is transformed into DH5 $\alpha$ , sequence-confirmed and then transformed into BL21 DE3 cells. Both cell types containing this plasmid were restored at -80°C.

**Overexpression of double-tagged RNase E**. Fig. 3.3,b shows the SDS-PAGE gel we ran after overexpressing for 4 hour. As shown, we haven't been able to overexpress this protein with a double tag. It infers that the RNase E with a double tag is not stable and is degraded as we can see overexpression of molecules with lower molecular weights.

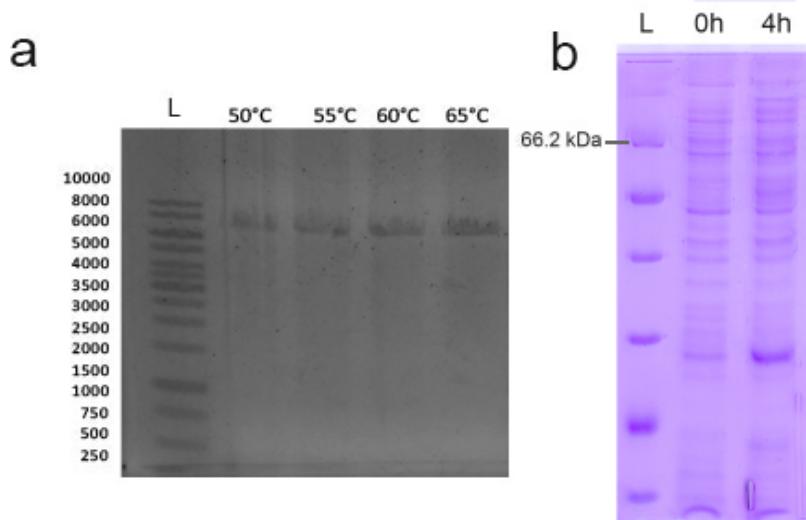


Figure 3.3: Left: Agorose gel electrophoresis run under 100 V for 35 minutes shows the PCR product containing truncated RNase E (7029bp) plus flag tag and his tag at different annealing temperatures. Right: SDS-PAGE showing the overexpression of truncated RNase E at before adding IPTG and 4hour after.

**Construction of a plasmid for RNAs.** Fig. 3.4 shows the DNA-PAGE gel results where it confirms that we have constructed the RNAs (ArcZ has 115bp and RprA has 105bp). Other than the bands for our RNAs we can see products with higher and lower molecular weights which might be because they haven't combined properly or the DNAs bind together. rprA1 is one of primers we used for constructing RprA gene which has 38 nucleotides. Then, we performed the gel extraction. Afterwards, to improve the transformation of this constructed plasmid it was phosphorylated and ligated as described in methods section. Then they are transformed into DH5 $\alpha$  cells, sequence-confirmed and transformed again inBL21 DE3 cells. The cells containing plasmids were restored in -80°C.

***In vitro* transcription of both RNAs were conducted successfully.** We first constructed by PCR the DNA templates for each of RNAs using corresponding forward and

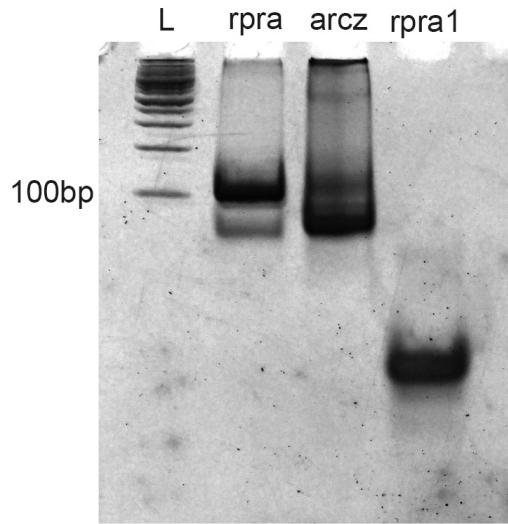


Figure 3.4: DNA-PAGE for construction of RprA and ArcZ genes run under 60V for 50 minutes. Rpr1 is one of oligonucleotides used in PCR.

reverse primers (see Appendix) (Fig. 3.5, the native gel for RprA). From the gel, we can see that we have a single band (RprA gene) when the annealing temperature is 70°C. We got the best result for ArcZ for annealing temperature of 62°C. Then we did in vitro transcrip-

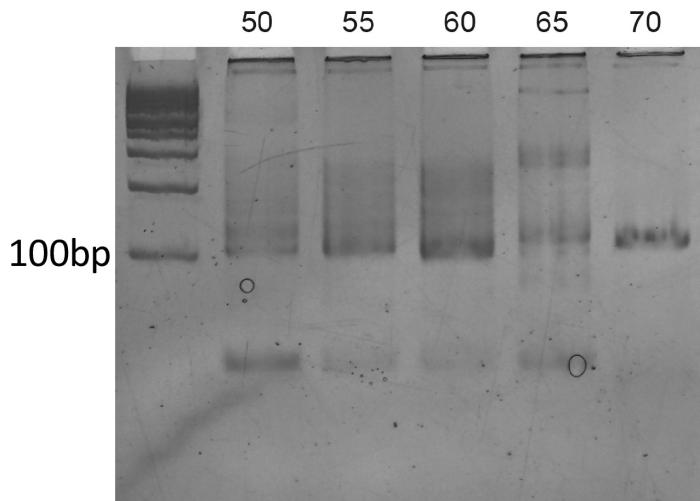


Figure 3.5: DNA-PAGE run under 60V for 60 minutes for different annealing temperatures. Clearly, we have a good results in 70°C. It was for construction of DNA template for in vitro transcription of RprA.

tion and produced RNAs. Fig. 3.6 shows a urea gel for the in vitro transcription products along time (18h is the overnight one). The smear of RNA in this gel might be due to a

plate separation. The higher molecular weight are possibly because of off-target products. Having these RNA smears confirms that we have obtained our RNA and we can go for the next step which is the purification of RNA.

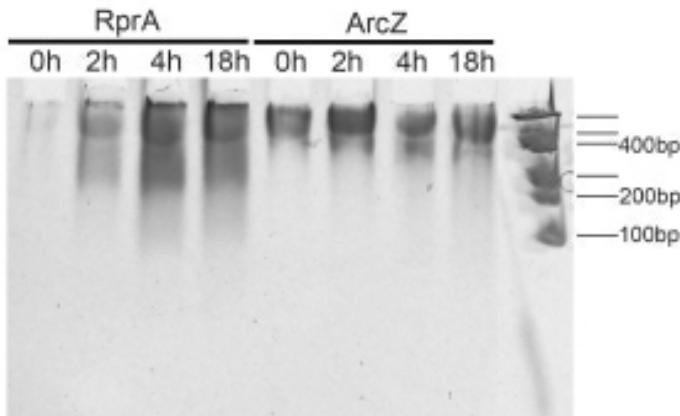


Figure 3.6: A urea gel for in vitro transcription products. However, it can verify that we have obtained the RNAs.

**ArcZ and RprA were purified using superdex75 column.** fig. 3.7 and fig. 3.8 show the purification results for RprA and arcz respectively. For RprA, the small peak on the left in the absorbance curve is found to be the RNA which was confirmed by running a urea gel on collected samples. We combined the samples containing RprA and measured the concentration by biodrop. It was  $\sim 4\mu\text{M}$ . Since the concentration was very low for labeling of the RNA, we start from the beginning and did purification process again and yielded a high concentration.

For ArcZ, in Fig. 3.8 from UV-visible spectroscopy, we found that again the first peak in absourbance curve was the RNA which was confirmed by running a urea gel for collected samples. We mised up the samples containing ArcZ and measured the concentration which was  $18.6\mu\text{M}$ . The RNAs stored in  $-20^{\circ}\text{C}$

**ArcZ was labelled with Pyrene.** For emission scan, ArcZ-pyrene with TAKM was excited at 350nm with a slit width of 1 nm. Then, fluorescence emission was monitored from 380 to 480nm. For Excitation scan, the emission wavelength was held at 395 and fluorescence excitation was monitored from 330 to 370nm. Fig. 3.9 shows the results

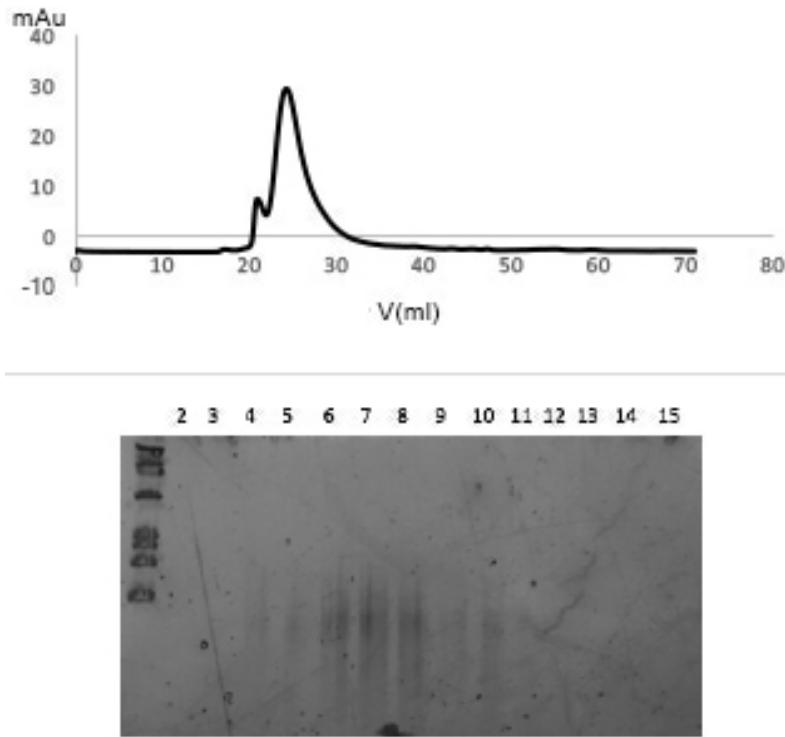


Figure 3.7: Superdex purification results for RprA. The bands in the left are for DNA ladder.

for excitation and emission scans of labbeled ArcZ which give us information about the excitation and emission wavelength of this labbled RNA. From this graph, we can see the the peak in excitation wavelength is 350nm and th peak in emission wavelength is 395nm.

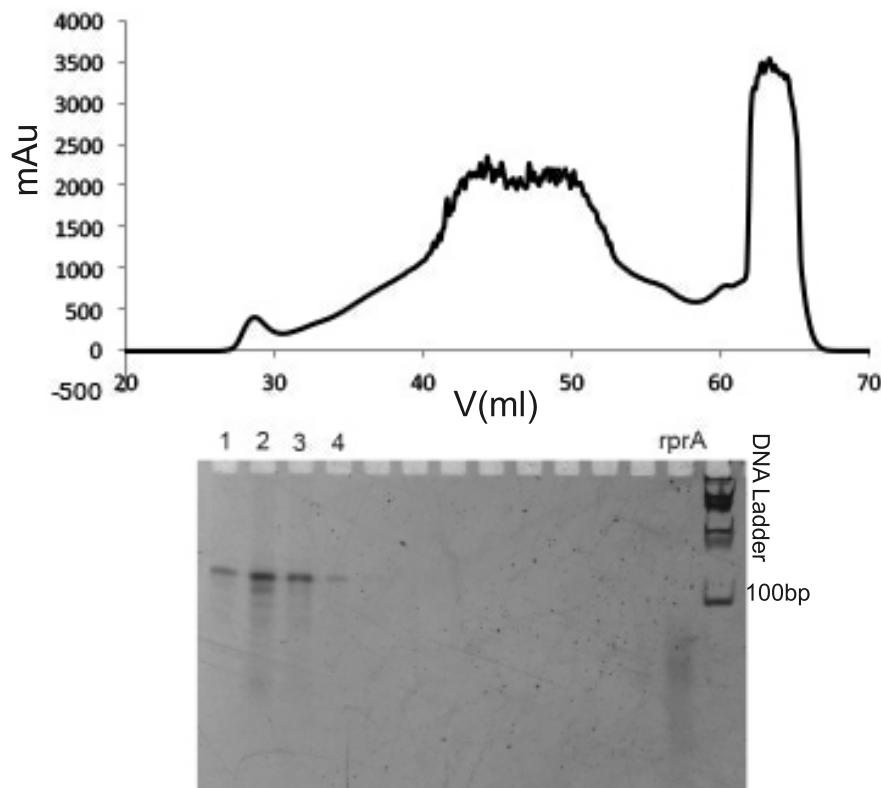


Figure 3.8: Superdex purification results for ArcZ.

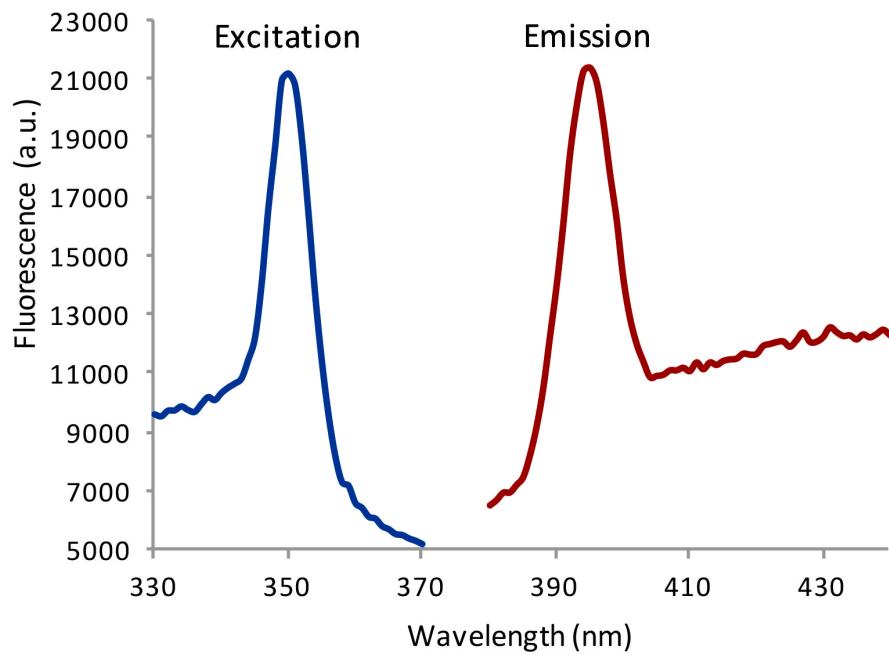


Figure 3.9: fluorescence spectroscopy results for the labbeled ArcZ.

## Discussion

For preparation of RNase E Δ529, we started by construction of a plasmid encoding the catalytic domain of RNase E. This truncation has a FLAG-tag on its C-terminal. We were successfully transformed it into two different cell strains of E.coli (BL21 DE3 and DH5 $\alpha$ ) and stored it at -80°C. We also performed the overexpression of the truncation RNase E. However, since FLAG-tag doesn't give us a high yield in protein purification, we construct a variation where we added HIS-tag to C-terminus of truncation RNase E. It was successfully inserted into the plasmid and transformed into E. coli. After trying three times overexpression, we weren't able to get the protein. we look at overexpression gels in previous chapter, we can see overexpression in lower molecular weight. We can conclude that the RNase E encoding gene with double-tag in C-terminus is not stable and gets degraded. In addition, since we are overexpressing the RNase E which is toxic for cells, we are not getting a high yield as well. For our next step, we are going to Add HIS-tag to N-terminus and will try overexpression again.

For preparation of RNAs (ArcZ and RprA), we started by construction the RNAs from corresponding primers and inserting them into pJET1.2/blunt plasmid. We successfully transformed the plasmid into E. coli cells and stored them at -80°C. Then, we performed in vitro transcription for both RNAs and then purified them. We performed labelling of ArcZ by Pyrene and obtained the emission and excitation wavelength of this RNA. For the next step we will label RprA.

After overexpression of truncation RNase E with double tag and purification of this protein, we will perform stopped-flow experiment to understand the kinetics of sRNA cleavage by RNase E. It would possibly give us information about association rate, dissociation rate, rate of conformational changes in backbone of RNA and in general rate of cleavage by RNase E.

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## Supplementary information

Table A.1: The primers used for constructing arcz and rpra genes

primers	Sequences
Oligo-rpra1	5'-GAC GGT TAT AAA TCA ACA CAT TGA TTT ATA AGC ATG GA-3'
Oligo-rpra2	5'-GTT TCA CTC AGG GGA TTT CCA TGC TTA TAA ATC-3'
Oligo-rpra3	5'-CCC CTG AGT GAA ACA ACG AAT TGC TGT GTG TAG TCT TTG CCC-3'
Oligo-rpra4	5'-AAA AAG CCC ATC GTA GGA GAC GGG CAA AGA CTA CAC AC-3'
Oligo-arcz1	5'-GTG CGG CCT GAA AAC AGG ACT GCG CCT TTG ACA TCA TC-3'
Oligo-arcz2	5'-GGG AAA TCG TGG CTG CGC CGT GCT TAT TAT GAT GAT GTC AAA GGC-3'
Oligo-arcz3	5'-GCC ACG ATT CTG GTG TTG GCG CAG TAT TCG CGC ACC CC-3'
Oligo-arcz4	5'-AAA AAT GAC CCC GGT TTG ACC GGG GTG CGC GAA TAC TGC GCC-3'

Table A.2: The primers used for construction of DNA templates for in vitro transcription of RNAs

primers	Sequences
For-rpra	5'-TAA TAC GAC TCA TAG GGA GAG ACG GTT ATA AAT CAA CAC ATT GAT TTA TAA GC-3'
Rev-rpra	5'-AAA AAT GAC CCC GGT TTG ACC GGG GTG CGC GAA TAC TG-3'
For-arcz	5'-TAA TAC GAC TCA CTA TAG GGA GAG TGC GGC CTG AAA ACA GGA CTG CGC C-3'
Rev-arcz	5'-AAA AAT GAC CCC GGT TTG ACC GGG GTG CGC GAA TAC TGC GCC AAC-3'

Table A.3: The primers used for construction of RNase E Δ529

primers	Sequences
For-RNase E	5'-GTA AGC GTC CGG AAC AAC CTG CGC TGT GAC CT T TGC CAT GCC GGA TGT G-3'
Rev-RNase E	5'-CAC ATC CGG CAT GGC AA A GGT CAC AGC GCA GGT TGT TCC GGA GGT TGT TCC GGA CGC TTA C-3'