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## Chapter **Y**

### **Assay of *Bacillus subtilis* ribonuclease activity *in vitro***

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**Running head:** *In vitro* assay of *B. subtilis* RNases

## Summary

Ribonucleases can cleave RNAs internally in endoribonucleolytic mode or remove one nucleotide at a time from either the 5' or 3' end through exoribonuclease action. To show direct implication of an RNase in a specific pathway of RNA maturation or decay requires the setting up of *in vitro* assays with purified enzymes and substrates. This chapter complements the previous chapter X on assays of ribonuclease action *in vivo* by providing detailed protocols for the assay of *B. subtilis* RNases with prepared substrates *in vitro*.

**Keywords:** Ribonuclease, protein purification, RNA substrates, *in vitro* transcription, radioactive labelling.

## 1. Introduction

The Gram-positive model organism *Bacillus subtilis* has >20 different ribonucleases (RNases) involved in mRNA and stable RNA maturation and decay [1]. Remarkably, fewer than half of these RNases overlap with the well-studied *E. coli* model. In *B. subtilis*, the main endoribonucleases involved in mRNA decay are RNase Y, which cleaves in single stranded AU-rich regions, and RNase III, which cleaves dsRNA. The main 5'-exoribonuclease is RNase J1, which functions in a stoichiometric complex with its paralog RNase J2, while the main 3'-exoribonucleases are PNPase, RNase PH, RNase R and YhaM. rRNA processing involves RNase III, RNase J1, YqfG, Mini-III and RNase M5 in *B. subtilis*, while tRNA processing requires RNase P and either RNase Z or the four 3'-exoribonucleases, depending nominally on whether the tRNA has an encoded CCA.

RNases are routinely assayed *in vitro* to definitively demonstrate that the effect of an enzyme observed *in vivo* is direct rather than indirect (see chapter X). *In vitro* assays are also used to establish an enzyme's biochemical ( $K_m$ ,  $K_d$ ) or kinetic parameters ( $K_{cat}$ ). We generally use purified His-tagged enzymes for these assays. For new uncharacterized enzymes, we typically start with standard buffer at neutral pH, 100 mM NaCl, 10 mM  $Mg^{2+}$  as the most likely metal ion and a reducing agent (e.g. dithiothreitol [DTT]/ $\beta$ -mercaptoethanol) and then optimize each of these buffer components in turn. Although assays can be performed on generic RNA targets, it is preferable that the true substrates be used. Our standard operating procedure is to synthesize specific RNA substrates by transcribing PCR products containing an integrated phage T7 promoter. These can be labelled with  $\alpha$ - $^{32}P$ -UTP directly or can be synthesized as unlabelled substrates and labelled later at their 5' or 3' extremities using  $\gamma$ - $^{32}P$ -ATP or  $\alpha$ - $^{32}P$ -pCp. For very short RNA substrates (< 50 nucleotides [nts]), we order chemically synthesized RNA oligonucleotides and label these at their 5' or 3' extremities. Strategies also exist for non-radioactive labelling, for example using fluorescent or digoxigenin labelled probes, although we do not use these very often, because they are less sensitive than radioactively labelled substrates

In this companion chapter to chapter X, we describe how we typically assay RNase activities *in vitro*. We describe in depth how we prepare His-tagged RNases, their RNA substrates and finally how we set up the *in vitro* assays themselves. We provide detailed notes on all our lab secrets to succeed with these procedures.

## 2. Materials

## 2.1 Purification of His-tagged RNases

1. Incubator.
2. pET28-RNase plasmid for overexpression of the 6xHis-tagged protein of interest. The example given in this chapter, RNase J1, is prepared as described previously [2].
3. *E. coli* overproducing strain, e.g. BL21 Codon-Plus( $\lambda$ DE3)-pRIL (Stratagene) containing plasmid pRIL expressing rare tRNAs adapted for expression of *B. subtilis* proteins and transformed with plasmid pET28-RNase.
4. Growth medium: sterile 2XYT medium containing 50  $\mu$ g/mL Kanamycin and 25  $\mu$ g/mL Chloramphenicol.
5. 1 M Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) in sterile water. Store at -20°C.
6. Latex gloves or equivalent.
7. 0.22  $\mu$ m vacuum filters (500 mL - 1L)
8. Vacuum pump
9. Spectrophotometer.
10. French press (e.g. Glen Mills).
11. High-speed centrifuge equipped with JA-10 and JA-20 rotors (Beckman), or equivalent.
12. 250 mL centrifugation bottles.
13. 15 mL and 50 mL conical tubes.
14. Sterile 1.5 mL microcentrifuge tubes.
15. 10 mg/mL DNase I: dissolve 100 mg of lyophilized DNase I in 10 mL of a buffer containing Tris-HCl pH 7.8, 50 mM NaCl, and 20% (v/v) glycerol. Store in 500  $\mu$ L aliquots at -20°C.
16. Complete EDTA-free anti-protease tablets (Roche). Store at 4°C.
17. Basic FPLC system (e.g. GE Healthcare AKTA Start).
18. 0.5 M imidazole in deionized water, buffered with HCl to pH 7.8.
19. Commercial 1 mL His-Trap FF or Co-Talon columns (GE Healthcare) (see **Note 1**).
20. 500 mL Binding buffer: 20 mM Tris-HCl pH 7.8, 0.5 M NaCl, 0.1 % (v/v) Triton X-100, 10 % (v/v) glycerol. Filter at 0.22  $\mu$ m. Store at 4°C.
21. 500 mL Wash buffer: 20 mM Tris-HCl pH 7.8, 0.3 M NaCl, 20 mM Imidazole (see **Note 2**). Filter at 0.22  $\mu$ m. Store at 4°C.
22. 500 mL Elution buffer: 20 mM Tris-HCl pH 7.8, 0.3 M NaCl, 250 mM Imidazole (see **Note 2**). Filter at 0.22  $\mu$ m. Store at 4°C.

23. 1 L Dialysis buffer: 20 mM Tris-HCl pH 7.8, 0.3 M NaCl, 10% v/v glycerol, 0.5 mM dithiothreitol (DTT). Filter at 0.22  $\mu$ m. Store at 4°C.
24. Kit for measuring protein concentration (e.g. Protein assay kit II from Biorad).
25. Dialysis chambers or tubing.
26. Centrifugal filter concentration units (e.g. Amicon units from Millipore) with molecular weight cutoff adapted to the RNase of interest.

## **2.2 Protein gels (SDS-PAGE)**

1. Gel electrophoresis apparatus with gel casting system (e.g. Biorad Mini Protean 2) and power supply.
2. Dry temperature block.
3. Orbital shaker.
4. Latex gloves or equivalent.
5. Spatula
6. Tissue paper
7. 95% ethanol.
8. Isobutanol.
9. 40% acrylamide:bis-acrylamide (37.5:1) solution.
10. 1X Running buffer: 0.025 M Tris, 0.192 M glycine, 0.1 % (w/v) sodium dodecyl sulfate (SDS) in deionized H<sub>2</sub>O.
11. 4X Resolution buffer: 1.5 M Tris-HCl pH 8.8, 0.4 % (w/v) SDS.
12. 4X Stacking buffer: 0.5 M Tris-HCl pH 6.8, 0.4 % (w/v) SDS.
13. 10% acrylamide solution: for 10 mL, mix 2.5 mL of 4X resolution buffer with 2.6 mL of 40% acrylamide:bis-acrylamide (37.5:1) solution. Complete to 10 mL with deionized H<sub>2</sub>O. Vortex the solution.
14. 5% acrylamide solution: for 5 mL, mix 1.25 mL of 4X stacking buffer with 0.625 mL of 40% acrylamide:bis-acrylamide (37.5:1) solution. Complete to 5 mL with deionized H<sub>2</sub>O. Vortex the solution.
15. 10% (w/v) ammonium persulfate (APS). Make 1 mL aliquots and store at -20 °C.
16. N,N,N',N'-Tetramethylethylenediamine (TEMED).
17. 5X Gel loading buffer: 0.312 M Tris-HCl pH 6.8, 10 % (w/v) SDS, 0.5 M DTT, 50% (v/v) glycerol, 0.04 % (w/v) bromophenol blue.
18. Plastic containers for gel staining.

19. Staining solution: 0.2% (w/v) PhastGel Blue R (GE Healthcare), 30% (v/v) methanol, 10% (v/v) acetic acid in deionized H<sub>2</sub>O.
20. Fast Destaining solution: 50% (v/v) ethanol, 10% (v/v) acetic acid in deionized H<sub>2</sub>O.
21. Slow Detaining solution: 10% (v/v) ethanol, 10% (v/v) acetic acid in deionized H<sub>2</sub>O.
22. Conservation solution: 5% (v/v) acetic acid in deionized H<sub>2</sub>O.

### ***2.3 Preparation of substrates for RNase assays***

#### ***2.3.1 Preparation of labelled and unlabelled RNA substrates by T7 in vitro transcription***

1. Water bath.
2. Dry temperature block
3. Cold room
4. Dark room
5. Vortex
6. Screens and protective clothing; to work with <sup>32</sup>P.
7. Latex gloves
8. Paper towels
9. 0.22 µm vacuum filters (1L)
10. Vacuum pump
11. G50 spin columns (GE Healthcare), or equivalent.
12. Benchtop microcentrifuge.
13. Sterile, RNase free 1.5 mL microcentrifuge tubes.
14. Gel electrophoresis system with 40 x 34 cm glass plates, 0.4 mm spacers and 32-well gel comb for polyacrylamide gel electrophoresis.
15. Power supply.
16. 5% acrylamide solution: dissolve 420 g urea in 125 mL 40 % acrylamide:bis-acrylamide (19:1) solution and 100 mL 10X TBE buffer (from commercial source). Complete to 1L with RNase-free water. Filter solution with a 0.22 µm filter and keep at 4°C in a dark bottle. For 20% solution, use 500 mL 40 % acrylamide:bis-acrylamide
17. Chemical fume hood
18. Silanization solution to pre-treat front gel plate (Sigma).
19. 10% (w/v) APS in water. Make 1 mL aliquots and store at -20 °C.
20. TEMED.
21. 1X TBE buffer, for electrophoretic transfer buffer. Prepare 1 L solution by dilution of 100 ml of 10X TBE buffer in 900 mL of deionized water.

22. 2X RNA loading dye (Ambion).
23. Saran Wrap™, or equivalent
24. X-ray film and film developer (e.g. Curix 60 AGFA).
25. 0.1 - 0.2 µg linear DNA template for *in vitro* T7 transcription.
26. Mega(Short)Script *in vitro* transcription kit (ThermoFisher).
27. Sterile RNase-free water.
28. 20 U/µL T7 RNA polymerase with 5X Transcription buffer.
29. 1U/µL RQ1 DNase (Promega).
30. 40 U/µL RNasin (Promega).
31. rNTP Mix (2.5 mM ATP, CTP and GTP; 60 µM UTP).
32. Radionuclides:  $\alpha$ -<sup>32</sup>P-UTP;  $\alpha$ -<sup>32</sup>P-pCp;  $\gamma$ -<sup>32</sup>P-ATP
33. 100 mM GMP
34. 100 mM Guanosine
35. 100 mM DTT.
36. Quick Calf intestinal alkaline phosphatase (CIP).
37. 10X CutSmart buffer (NEB).
38. 10 M LiCl.
39. 20 mg/mL glycogen in sterile, RNase-free water.
40. 10 U/µL T4 RNA ligase 1 and 10X T4 RNA ligase buffer.
41. 10 mM ATP.
42. Dimethyl sulfoxide (DMSO).
43. 10 U/µL T4 polynucleotide kinase (PNK) and 10X T4 PNK kinase buffer.
44. Water-saturated phenol pH 5.
45. New scalpel and clean (ethanol-flamed) forceps.
46. Rotating wheel for mixing samples
47. Cold-room (4°C)
48. 95% ethanol, stored at -20°C.
49. 70% ethanol, stored at -20°C.
50. UV-vis spectrophotometer with microliter capability.

### **2.3.2 Preparation of ribosome precursors**

1. Sterile 2XYT growth medium plus appropriate antibiotics
2. Items 6 - 15 from Section 2.1.



3. Benchtop microcentrifuge.
4. Cold room
5. Vortex (with a gentle shaking mode).
6. Ultracentrifuge (Beckman) and preparative swinging bucket (SW28) rotor.
7. Ultracentrifuge tubes for SW28 rotor: open-top polyclear tubes (Seton).
8. Gradient maker (Biocomp).
9. Piston gradient fractionator (Biocomp).
10. Benchtop ultracentrifuge with TLA 100.3 rotor (Beckman).
11. TLA 100.3 polycarbonate centrifuge tubes (Beckman).
12.  $\beta$ -Mercaptoethanol.
13. 5X Buffer A: 100 mM Tris-HCl pH 7.5, 1 M  $\text{NH}_4\text{Cl}$ , 15 mM  $\text{MgCl}_2$ . Filter at 0.22  $\mu\text{m}$ . Store at 4°C.
14. 1X buffer A, obtained by diluting the 5X buffer A stock in sterile deionized  $\text{H}_2\text{O}$  and adding 6 mM  $\beta$ -Mercaptoethanol. Prepare fresh (see **Notes 3, 4**). Filter at 0.22  $\mu\text{m}$ .
15. 10% (w/v) high-grade sucrose in 1X Buffer A. Prepare fresh (see **Notes 3, 4**). Filter at 0.22  $\mu\text{m}$ .
16. 30% (w/v) high-grade sucrose in 1x Buffer A. Prepare fresh (see **Note 3, 4**). Filter at 0.22  $\mu\text{m}$ .
17. 10 mg/mL DNase I: dissolve 100 mg of lyophilized DNase I in 10 mL of a buffer containing Tris-HCl pH 7.8, 50 mM NaCl, and 20% (v/v) glycerol. Store in 500  $\mu\text{L}$  aliquots at -20°C.
18. Liquid nitrogen and long insulated gloves for handling it safely.

### **2.3.3 RNase assays *in vitro***

1. Sterile RNase-free microcentrifuge tubes.
2. Gel electrophoresis system with 40 x 34 cm glass plates, 0.4 mm spacers and 32-well gel comb for polyacrylamide gel electrophoresis.
3. Power supply.
4. Screens and protective clothing to work with  $^{32}\text{P}$ .
5. PhosphorImager with  $^{32}\text{P}$ -phosphorimaging screen and cassette (GE Healthcare).
6. Mini-sub-cell system (BioRad) with gel tray, comb, tank, and gel mold, or equivalent system for the preparation and migration of agarose gels (15 x 7 cm).

7. 1% (w/v) ethidium bromide in water. Handle reagent, solutions, waste, etc. with great care, following adequate CMR safety procedures.
8. 10X Tris-Borate-EDTA (TBE) buffer. Buy RNase-free stock from reliable commercial source.
9. 1X TBE running buffer: Prepare 1L solution by dilution of 100 ml of 10X TBE stock with 900 ml of RNase-free water.
10. 1% (w/v) Agarose gel. Add 1 g high-grade agarose to 100 mL of 1X TBE buffer. Heat mixture in a microwave oven to dissolve agarose and then pour it in a 15 x 7 cm gel tray installed in gel caster. Add comb and let gel solidify for 30 min at room temperature.
11. Ice bucket.
12. Spatula.
13. Water bath.
14. Dry temperature block.
15. Saran wrap<sup>TM</sup>, or equivalent.
16. Purified RNase (see Section 3.2).
17. Enzyme Dilution buffer: 20 mM Tris-HCl pH 7.8, 0.3 M NaCl, 10% v/v glycerol, 0.5 mM dithiothreitol (DTT).
18. 5X RNase J1 buffer: 100mM Tris-HCl pH 8, 40 mM MgCl<sub>2</sub>, 500mM NH<sub>4</sub>Cl, 0.5mM DTT.
19. RNA substrate: 20 nts synthetic oligoribonucleotide, 5' or 3' radiolabeled (see Section 3.3.4) and gel purified (see Section 3.3.5).
20. Nuclease-free water
21. 20% acrylamide solution: dissolve 420 g urea in 500 mL 40 % acrylamide:bis-acrylamide (19:1) solution and 100 mL 10X TBE buffer (from commercial source). Complete to 1L with RNase-free water. Filter solution on a 0.22 µm filter and keep at 4°C in a dark bottle.
22. Solutions 18 - 22 from Section 2.3.1.
23. Whatman 3 MM paper cut to the size of the gel.
24. Vacuum gel dryer and vacuum pump.

### **3. Methods**

#### ***3.1 Cultures for overproduction of His-tagged proteins***

Affinity tags are highly efficient tools for purifying proteins. One of the most used is the His-tag, which provides good yields of tagged protein from inexpensive, high capacity resins. We commonly use Nickel or Cobalt Talon (Co-Talon) affinity metal resins, depending on the protein solubility with respect to pH (see **Note 1**). Both can be employed in native or denaturing conditions (see **Notes 5, 6**).

1. Inoculate 20 mL of growth medium with the *E. coli* RNase overproducing strain.
2. Incubate at 37°C overnight with shaking.
3. Use 4 mL of overnight culture to inoculate 400 mL of fresh growth medium and grow at 37°C with shaking to an optical density at 600nm (OD<sub>600</sub>) of 0.4-0.6 (see **Notes 7, 8**).
4. To induce protein expression, add 200 µL of 1M IPTG (0.5 mM, final concentration) and incubate at 37°C under shaking for 3-4 h.
5. Pellet cells in 250 mL centrifuge bottles for 20 min at 9,000 g at 4°C.
6. Pour off all but about 20 mL of supernatant.
7. Carefully dislodge the pellet with a spatula and transfer pellet and remaining supernatant to a 50 mL conical tube.
8. Centrifuge for 15 min at 5,000 g at 4°C.
9. Remove supernatant and store the pellet frozen at -20°C until further use.

### ***3.2 Purification of His-tagged proteins under native conditions***

Wear gloves to avoid contamination of protein samples.

1. Resuspend the frozen cells in 10 mL of Binding buffer at 4°C.
2. Add 10 µL 10 mg/mL DNase I and an EDTA-free anti-protease tablet.
3. Before making cell lysate, install the His-Trap FF on the FPLC system and start equilibration of column with Binding buffer at 1 mL/min (see **Note 9**).
4. Pass the suspension (from step 2) twice through a French press (20,000 psi).
5. Centrifuge the lysate for 30 min at 15,000 g and keep supernatant.
6. Add 1/500 volume of 0.5 M imidazole-HCl (pH 7.8) to the supernatant to give a final concentration of 1 mM.
7. Apply the resulting mix to the column at the same flow rate as during equilibration.
8. Wash the column with approximately 10 mL Binding buffer. Collect this fraction in a 15 mL tube for analysis.
9. Wash with 15 mL Wash buffer. Collect this fraction in a 15 mL tube for analysis (see **Note 2**)

10. Apply 10 mL Elution buffer to the column and collect 1 mL fractions in 1.5 mL microtubes (see **Notes 2, 10**).
11. Confirm the protein peak by measuring protein concentration using a dedicated kit. Keep small aliquots (e.g. 50  $\mu$ L) of these fractions for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Section 3.3).
12. Pool the remainder of the peak fractions and dialyze immediately overnight against Dialysis buffer (see **Note 11**).
13. Concentrate samples if necessary with filter concentration units, following manufacturer's instructions.
14. Analyze different fractions of the purification scheme by SDS-PAGE.
15. Measure protein concentration with a dedicated kit, aliquot and store at  $-80^{\circ}\text{C}$ .

### **3.3 Protein gels (SDS-PAGE)**

SDS-PAGE is a technique designed to dissociate proteins into their individual polypeptide subunits by denaturation in the presence of detergent (SDS). Since the charge contribution is negligible compared to that of the negatively charged SDS, it is also possible to determine the molecular weight of the polypeptides by comparing their migration position to that of a molecular weight standard.

1. Clean the glass plates with a few milliliters of 95% ethanol and assemble the 1 mm wide, (10 x 8) cm gel cassette, following manufacturer's instructions.
2. Mix 10 mL of the 10% polyacrylamide gel solution with 50  $\mu$ L 10% APS and 10  $\mu$ L TEMED and pour this "resolution gel" mixture into the gel cassette.
3. Layer a few drops of isobutanol to the unpolymerized gel to ensure the top surface of the gel is flat.
4. Polymerize the gel for 1 h at room temperature.
5. Wash the gel surface thoroughly with deionized water to remove isobutanol.
6. Mix 5 mL of the 5% polyacrylamide gel solution with 25  $\mu$ L 10% APS and 10  $\mu$ L TEMED.
7. Pour this "stacking gel" mixture on top of the resolution gel and insert the comb immediately, without introducing air bubbles.
8. Polymerize the gel for 1 h at room temperature.
9. Mount gel on apparatus and fill reservoirs with 1X Running buffer.

10. Mix 4 volumes of protein sample with 1 volume of 5X gel loading buffer. The final volume should not exceed 25  $\mu$ L.
11. Heat the samples for 3 min at 90°C in a dry temperature block and load them in the gel wells.
12. Run the gel at room temperature at 20 mA. Stop the migration when the bromophenol blue dye reaches the bottom of the gel.
13. Separate the gel plates with the help of a spatula and place the gel in Staining solution in a plastic container.
14. Shake slowly on orbital shaker for 30 min.
15. Remove Staining solution and rinse the gel with deionized water.
16. Add 20 mL of the fast Destaining solution and shake slowly for 1 h.
17. Remove fast Destaining solution and rinse the gel with deionized water.
18. Add 20 mL of the slow Destaining solution and shake slowly for a few hours until the desired color is obtained (see **Note 12**).
19. Store gel in Conservation solution.

### ***3.3 Preparation of substrates for RNase assays***

All materials and solutions must be RNase-free and gloves should be worn for the entirety of all procedures.

#### ***3.3.1 T7 in vitro transcription***

We generally use PCR templates with an integrated T7 RNA polymerase promoter as a DNA template for *in vitro* transcription reactions. The minimal sequence of T7 RNA polymerase promoter is 5'-ATTAATACGACTCACTATAG-3' for transcripts that start with a G and 5' CAGTAATACGACTCACTATTA 3' for transcripts that start with an A. To increase the yield of *in vitro* transcription, add one or two extra guanines to the 3' end of the minimal promoter sequence, e.g. **GGG** or **AGG**, but it is important to note that this addition will be incorporated into the 5' end of the transcript. Linearized plasmid DNA that contains a T7 RNA polymerase promoter site can also be used as a template for *in vitro* transcription.

#### ***3.3.2 Production of unlabelled T7 in vitro transcripts with various 5' ends.***

We have adapted the MEGA(Short)Script kit protocol to obtain different 5' ends (5' triphosphate, 5'-monophosphate, 5'-hydroxyl) on RNA transcripts. For short transcripts (<150 nts), we use the MEGAShortScript kit; for those that are longer, we use the MEGAScript kit.

1. Prepare transcription mixture according to the following table in 1.5 mL microtubes (see **Notes 13, 14**).

	Synthesized RNA		
	5' tri-phosphate	5'-monophosphate	5'-hydroxyl
Nuclease-free water	6	4.5	4.5
10x reaction buffer	2	2	2
75 mM ATP	2	2	2
75 mM UTP	2	2	2
75 mM CTP	2	2	2
75 mM GTP	2	0.5	0.5
100 mM GMP	0	3	0
100 mM Guanosine	0	0	3
1 µg/µl DNA template	2	2	2
T7 RNA polymerase	2	2	2
Total volume	20 µl	20 µl	20 µl

2. Incubate mixture for at least 3h at 37°C in a water bath (see **Note 15**).
3. Add 2 µl of TURBO DNase (from the MEGA(Short)Script kit), then incubate for 15 minutes at 37°C to degrade the DNA template.
4. Clean up unincorporated nucleotides on a G50 spin column (following manufacturer's recommendations) or gel purify the RNA transcript (Section 3.3.6).
5. Determine RNA concentration from UV absorbance at 260 nm using an extinction coefficient of  $0.025 (\mu\text{g/ml})^{-1} \text{ cm}^{-1}$ .

### 3.3.3 Production of $\alpha$ -<sup>32</sup>P-UTP labelled T7 in vitro transcripts

To produce uniformly labelled transcripts, we add a lower concentration of cold UTP (60µM) while keeping the rest of rNTPs at 2.5mM (see **Note 16**). Since the yields required are typically not that high, we use individually purchased enzymes and nucleotides rather than the more expensive kits for this purpose. All radioactive materials should be handled by properly trained scientists, following safety rules and local regulations.

1. In a sterile, RNase-free 1.5 mL microtube, mix 0.1 - 0.2 µg linear DNA template, 2.5 µL 100 mM DTT, 5 µL of rNTP label mix (2.5 mM each ATP, CTP GTP and 60 µM UTP),

4  $\mu\text{L}$   $\alpha$ - $^{32}\text{P}$ -UTP, 1.5  $\mu\text{L}$  40 U/ $\mu\text{L}$  RNasin, 5  $\mu\text{L}$  5X T7 transcription buffer, 2  $\mu\text{L}$  20 U/ $\mu\text{L}$  T7 RNA polymerase, qsp 25  $\mu\text{L}$  with RNase-free sterile water (see **Note 13**).

2. Incubate for 1h30 in a water bath at 37°C
3. Add 2  $\mu\text{L}$  1U/  $\mu\text{L}$  RNase-free RQ1 DNase and incubate for 15 min at 37°C.
4. Clean up unincorporated nucleotides on a G50 spin column (following manufacturer's recommendations) or gel purify the RNA transcript (section 3.3.6).

### 3.3.4 5' labelling of RNA substrates

Unlabelled *in vitro* transcribed RNAs or custom synthesized RNA oligonucleotides can be labelled at the 5' end using T4 PNK and  $\gamma$ - $^{32}\text{P}$ -ATP, or at the 3' end using RNA ligase and  $\alpha$ - $^{32}\text{P}$ -pCp, and used as substrates for RNase assays. *In vitro* transcribed RNAs bearing a 5'-triphosphate must be dephosphorylated prior to 5' labelling by treatment with CIP. To remove 5' tri-phosphate, proceed to step 1. If the RNA has been synthesized with a 5'OH group, proceed to step 11.

1. Mix 2  $\mu\text{L}$  of 10X CutSmart buffer, 1  $\mu\text{L}$  of RNasin, 2  $\mu\text{L}$  of CIP, 10 to 150 pmol of unlabeled RNA (from Section 3.3.2, step 5), and adjust volume to 20  $\mu\text{L}$  with RNase-free sterile water.
2. Incubate mixture for 30 min at 37°C.
3. Add 180  $\mu\text{L}$  of RNase-free sterile water, 10  $\mu\text{L}$  of LiCl and 200  $\mu\text{L}$  of water-saturated phenol pH 5.
4. Vortex 1 min at maximum speed.
5. Centrifuge for 10 min at 16 000g at 4°C.
6. Transfer aqueous phase to fresh tube and add 1  $\mu\text{L}$  of glycogen and 600  $\mu\text{l}$  (3 volumes) of 95% ethanol (stored at -20°C) and incubate for at least 30 min at -20°C.
7. Centrifuge for 30 min at 16 000 g at 4°C. Discard the supernatant.
8. Wash pellet with 400  $\mu\text{L}$  of 70% ethanol stored at -20°C to remove traces of salt.
9. Centrifuge for 5 min at 16 000 g at 4°C. Discard the supernatant and repeat the wash step with 95% ethanol.
10. Air dry the pellet and resuspend it in 10  $\mu\text{L}$  RNase-free water. Determine RNA concentration from UV absorbance at 260 nm using an extinction coefficient of 0.025 ( $\mu\text{g}/\text{ml}$ ) $^{-1}$   $\text{cm}^{-1}$ .

11. In a sterile, RNase-free 1.5 mL microtube, mix 10 to 40 pmol dephosphorylated RNA, 2  $\mu\text{L}$  10X T4 PNK kinase buffer, 3  $\mu\text{L}$   $\gamma$ - $^{32}\text{P}$ -ATP, 9  $\mu\text{L}$  RNase-free water, 2  $\mu\text{L}$  T4 PNK kinase.
12. Incubate 30 min at 37°C in a water bath.
13. Clean up unincorporated nucleotides on a G50 spin column (following manufacturer's recommendations) or gel purify  $^{32}\text{P}$ -labeled RNA (Section 3.3.6).

### **3.3.5 3'-end labelling of RNA substrates**

1. In a sterile, RNase-free 1.5 mL microtube, mix 10  $\mu\text{L}$  10  $\mu\text{M}$  RNA (from Section 3.3.2, step 5), 2.5  $\mu\text{L}$  DMSO, 1.25  $\mu\text{L}$  10 mM ATP, 2.5  $\mu\text{L}$  10x T4 RNA ligase buffer, 1.25  $\mu\text{L}$  40U/ $\mu\text{L}$  RNasin, 3  $\mu\text{L}$   $\alpha$ - $^{32}\text{P}$ -pCp, 2.5  $\mu\text{L}$  RNase-free sterile water, 2  $\mu\text{L}$  T4 RNA ligase.
2. Incubate for 4h at 16°C or overnight at 4°C.
3. Incubate the mixture for 15 min at 65°C in a dry temperature block to inactivate the T4 RNA ligase.
14. Clean up unincorporated nucleotides on a G50 spin column (following manufacturer's recommendations) or gel purify  $^{32}\text{P}$ -labeled RNA (Section 3.3.6).

### **3.3.6 Gel purification of RNA**

Non-homogenous PCR templates, abortive T7 RNA polymerase transcription products or incomplete chemical synthesis of RNA can lead to the appearance of labelled contaminating species that are shorter than the desired transcript. In this case, full-length (FL) labelled RNA can be purified from a polyacrylamide gel (from 5 to 20% depending on the size of the RNA).

1. To pour a vertical polyacrylamide gel (0.4 x 400 x 340 mm), treat the smaller glass plate with a few milliliters of silanization solution and spread evenly with a paper towel under a fume hood.
2. Mix 70 mL of the polyacrylamide gel solution with 700  $\mu\text{L}$  10% APS and 70  $\mu\text{L}$  TEMED. Pour gel in a preassembled 0.4 x 400 x 340 mm gel cassette and insert a 32-well gel comb immediately, without introducing air bubbles. Allow the gel to polymerize for 1 h at room temperature.
3. Mount gel on the electrophoresis apparatus and fill reservoirs with 0.5X TBE buffer.



4. Rinse the gel wells with a Pasteur pipette filled with 0.5X TBE to remove urea and polyacrylamide fragments.
5. Add the same volume of 2X RNA dye to the  $^{32}\text{P}$  labelled RNA samples and load all the sample, occupying more than one lane if necessary.
6. Run the gel at 60W to separate the full-length transcript from contaminating truncated species.
7. Remove one of the glass plates and cover the gel in Saran Wrap<sup>TM</sup> to prevent it from drying.
8. Expose the gel to an X-ray film in a dark room (1-10 minutes depending on efficiency of radioactive labelling), taking care to align the edge of the film with the top and one of the side edges of the glass plate. Fold down one corner of the film for orientation.
9. Develop the film.
10. Place the developed film with the visible radioactive band under the glass plate, in the correct orientation, aligning the film to the top and side edges as before, to precisely locate the portion of the gel to cut out.
11. Cut the band out (through the Saran Wrap<sup>TM</sup>) as tightly as possible with a clean new scalpel, peel off the Saran wrap and place the radioactive gel slice in 400  $\mu\text{L}$  of 0.3 M sodium acetate in a 1.5 mL microtube. This can all be done with the same scalpel and clean forceps.
12. Elute on a rotating wheel for 4h in the cold room.
13. To precipitate RNA, add 1 mL 95% ethanol, 1  $\mu\text{L}$  20 mg/ml glycogen and incubate for at least 1 hr at  $-20^{\circ}\text{C}$ .
14. Centrifuge for 30 min at 20 000 g at  $4^{\circ}\text{C}$ . Discard supernatant.
15. Add 200  $\mu\text{L}$  70% ethanol ( $-20^{\circ}\text{C}$ ) to pellet
16. Centrifuge for 30 min at 20 000 g at  $4^{\circ}\text{C}$ . Remove supernatant carefully.
17. Add 200  $\mu\text{L}$  95% ethanol ( $-20^{\circ}\text{C}$ ) to pellet
18. Centrifuge for 30 min at 20 000 g at  $4^{\circ}\text{C}$ . Remove supernatant carefully.
19. Resuspend RNA pellet in 20-50  $\mu\text{L}$  nuclease free water.

### ***3.3.7 Preparation of ribosome precursors***

In addition to assays using *in vitro* transcribed RNA substrates, RNase activity can be assessed on more complex substrates isolated *in vivo*. This is particularly useful for the study of RNases involved in ribosomal RNA (rRNA) maturation that recognize components of the ribosome. For example, RNase M5 cleavage is dependent on prior binding of L18 to the pre-

5S rRNA whereas Mini III requires L3 as a cofactor for efficient maturation of the 23S rRNA [3,4]. *In vivo* isolation of precursor ribosome subunits allows one to perform *in vitro* assays of rRNA maturation enzymes with their natural substrates. Precursor ribosome subunits are purified from strains lacking the relevant maturation enzymes using the ultracentrifugation method described below, and used as cold substrates for *in vitro* RNase cleavage assay. The subsequent rRNA maturation is assessed *via* Northern blot using rRNA specific probes (see chapter X, section 3.3).

1. Inoculate *B. subtilis* RNase mutant strain of interest in 5 mL overnight cultures in 2XYT medium with appropriate antibiotics at 37°C with shaking.
2. Measure the OD<sub>600</sub> of the overnight culture.
3. Inoculate 260 mL of fresh 2XYT medium without antibiotics at a starting OD<sub>600</sub> of 0.05 and incubate at 37°C with shaking.
4. At OD<sub>600</sub> around 0.7-0.8, harvest 250 mL cells in centrifugation bottles by 10 min centrifugation at 9,000 g in a JA-10 rotor at 4°C (see **Note 17**). Discard supernatant.
5. Wash the cell pellet twice by resuspension in 50 mL ice cold 1X Buffer A (20 mM Tris-HCl pH 7.5, 200 mM NH<sub>4</sub>Cl, 6 mM β-Mercaptoethanol) (see **Note 3**), containing 3 mM or 10 mM MgCl<sub>2</sub> (see **Note 4**) in a 50 mL conical tube and centrifugation in a JA20 rotor for 10 min at 5,000 g at 4°C. Store the cell pellet at -20°C.
6. Resuspend the cell pellet in 1.7 mL cold 1X Buffer A and add 1.7 μL 10 mg/mL DNase I. Break open the cells by two passages through a chilled French press at 20,000 psi. Re-chill French press for a few minutes on dry ice every second bacterial sample.
7. Clarify the lysate by 30 min centrifugation in a benchtop centrifuge at 16,000 g to get rid of the cell debris (keep supernatant).
8. Prepare 10-30% (w/v) linear sucrose gradients by layering approx. 20 mL 10% sucrose solution on top of approx. 20 mL of the 30% sucrose solution in SW28 open top polyclear tubes (see **Note 18**), as described in the gradient maker user manual, and running the appropriate program in the Biocomp gradient maker (see **Note 19**).
9. Carefully remove 1 mL of sucrose solution from the top the gradient to make space for the sample. Gently load the entire supernatant from step 7 on top of the gradient.
10. Centrifuge at 64,600 g for 16 h in a SW28 rotor.
11. Collect 1 mL fractions with the Piston Gradient Fractionator, according to the manufacturer's instructions.

12. Pool fractions of interest according to the absorbance profile at OD<sub>260nm</sub> (see **Notes 20, 21**).
13. To concentrate precursor particles, centrifuge at 264,500 g in a TLA 100.3 rotor in polycarbonate centrifuge tubes.
14. The ribosomal pellet obtained after ultracentrifugation is transparent and very sticky, make sure not to touch it with the pipette tip while removing all the supernatant.
15. Resuspend pellet in 50 to 100 μL 1X Buffer A by vortexing gently in cold room for 20 min (or until all the ribosomal pellet is resuspended).
16. Freeze aliquots in liquid nitrogen and store at -80°C.

### 3.4 RNase assays *in vitro*

When doing *in vitro* assays, we typically first check the effect of different protein concentrations on the degradation/maturation of the substrate in a dose response assay. Once we have identified an appropriate enzyme concentration that functions well, we often switch to kinetic reactions to identify subtle differences between wild-type and mutant variants, for example. As substrates, we use radiolabeled RNAs, cold transcripts or more complex substrates such as ribosome precursors (see previous section). While the enzyme reactions can be followed directly on gels with labelled substrates, with unlabelled substrates, the assays are followed by Northern blots (see chapter **X**, section 3.3). Here, we detail a typical protocol to follow the release of a <sup>32</sup>P-labelled 5'-monophosphate by the *B. subtilis* 5'-exoribonuclease RNase J1 from a 20 nt RNA, as an example [5]. For other enzymes, the optimal buffer, substrate and general reaction conditions might be different.

#### 3.4.1 Dose-response assay

1. To find the optimal RNase J1 concentration for an *in vitro* assay, first prepare a dilution of the enzyme at 1 mg/mL using enzyme Dilution buffer in 1.5 mL microtubes and then make 10<sup>-1</sup>, 10<sup>-2</sup> and 10<sup>-3</sup> dilutions of this preparation for assays (see **Notes 22, 23**). Keep the tubes on ice while preparing the reaction mixtures. Each 10 μL reaction will contain 1 μL of diluted RNase J1, 0.5 μL 5'-labelled RNA, 2 μL 5X RNase J1 buffer, 6.5 μL nuclease-free water.
2. In a 1.5 mL RNase free microcentrifuge tube, prepare a reaction mix for n+1 samples (without the protein). For instance, mix 3.5 μL 5'-labelled RNA, 14 μL 5X RNase J1 buffer and 45.5 μL nuclease-free water for 6 samples (e.g. four reaction samples and two negative control samples).

3. Dispatch 9  $\mu\text{L}$  of this mixture into new microtubes (one per sample) on ice. Add 1  $\mu\text{L}$  of the protein (different concentrations) per tube or 1  $\mu\text{L}$  enzyme Dilution buffer for the negative controls (see **Notes 24, 25**).
4. Incubate 20 min at 37°C in a water bath.
5. Add 10  $\mu\text{l}$  2X RNA loading dye and store frozen (-20°C) until ready for loading.
6. Prepare a 20% polyacrylamide gel (0.4 x 400 x 340 mm) as in Section 3.3.6 (see **Notes 26, 27**).
7. Denature samples for 5 min at 90°C in a dry temperature block, and load 10  $\mu\text{L}$  of each sample in separate gel wells.
8. Run the gel at 60W in 1X TBE.
9. Separate gel plates carefully with a spatula. The gel should stick to the non-silanized plate.
10. Apply a dry sheet of Whatman 3 MM paper cut to the appropriate size to the gel (see **Note 28**).
11. Peel the paper and gel together from the gel plate and cover the gel surface in Saran Wrap™.
12. Sandwich between two additional sheets of Whatman 3MM paper and vacuum dry the gel for 1h30 at 80°C (see **Note 29**).
13. Place the gel in a PhosphoImager cassette and expose it to a  $^{32}\text{P}$  phosphorimaging screen. A typical dose-response profile is depicted in **Figure 1**. [\[Figure 1 near here\]](#)

### 3.4.2 Kinetic assay

Once the optimal protein concentration has been found, a kinetic assay can be done with different (n) time points for finer analysis of the protein and mutant variants. In this case, we prepare a mixture for n+1 time points with all components, and add the enzyme at T0. In a typical experiment, we remove 5  $\mu\text{L}$  samples after 2, 5, 10, 20, and 30 min.

1. Prepare n+1 microtubes containing 5  $\mu\text{L}$  2x RNA loading dye on ice.
2. Prepare a denaturing 20% polyacrylamide gel (see Section 3.3.6)
3. In 1.5 mL RNase-free microtube, mix 4  $\mu\text{L}$  5'-labelled RNA, 8  $\mu\text{L}$  5X RNase J1 buffer, and 24  $\mu\text{L}$  nuclease-free water (for 5 time points and two negative controls).
4. Remove 5  $\mu\text{L}$  of the step 3 mixture for the C0 control and add it to one tube from step 1. Keep on ice.
5. Transfer 5  $\mu\text{L}$  of the step 3 mixture into a new tube for the C30 control.

6. Add enough RNase J1 enzyme to the step 3 mixture for n-1 aliquots and mix. In the example given, this would correspond to 3  $\mu\text{L}$  (0.5  $\mu\text{L}$  per time point) at the appropriate dilution determined from the dose response curve in Section 3.4.1.
7. Incubate the C30 control and the reaction mixture (from step 6) at 37°C. Remove 5  $\mu\text{L}$  sample aliquots at 2, 5, 10, 20 and 30 min from the reaction mixture and add each aliquot to a tube from step 1. Store aliquots on ice. After 30 min of incubation, also add 5  $\mu\text{L}$  2X RNA loading dye to the C30 control and put on ice.
8. Incubate samples for 5 min at 90°C to denature them. Then load each 10  $\mu\text{L}$  sample into a well of the 20% polyacrylamide gel.
9. Run the gel at 60W in 1X TBE.
10. Process and dry gel as described in Section 3.4.1, steps 9-13 (see **Notes 28, 29**). A typical kinetic assay profile is depicted in **Figure 2**. [\[Figure 2 near here\]](#)

#### 4. Notes

1. Cobalt-Talon columns have been used to purify His-tagged proteins to a higher degree of purity than HisTrap columns in some cases. In this case the following buffers are used: Binding buffer: 50 mM sodium phosphate pH 7.0, 0.3 M NaCl, 0.1 % (v/v) Triton X-100, 10 % (v/v) glycerol; Wash buffer: 50 mM sodium phosphate pH 7.0, 0.3 M NaCl, 20 mM imidazole ; Elution buffer: 50 mM sodium phosphate pH 7.0, 0.3 M NaCl, 150 mM imidazole The protein can also be eluted from Co-Talon columns with a low pH elution buffer containing 50 mM sodium acetate pH 5.0, 0.3 M NaCl, and 150 mM imidazole.
2. If protein solubility permits, a more stringent wash and elution protocol involving a pH gradient instead of imidazole can be used. After binding, an alternative wash buffer (50 mM  $\text{NaH}_2\text{PO}_4$  pH 6.0, 0.3 M NaCl) is applied to the column, and the protein is eluted with 10 mL low pH elution buffer (50 mM sodium acetate pH 4.0, 0.3 M NaCl, 250 mM Imidazole).
3. Add  $\beta$ -Mercaptoethanol to Buffer A just before use.
4. 1X Buffer A is a typical ribosome storage buffer. The protocol given here, using 1X Buffer A with 3 mM  $\text{MgCl}_2$ , is designed to dissociate the small (30S) and large (50S) ribosomal subunits. 1X Buffer A with high magnesium concentration (10 mM  $\text{MgCl}_2$ ) is required to maintain assembled 70S ribosomes. Denaturing conditions can be used for proteins that form inclusion bodies or for soluble proteins whose His-tag is not accessible

for interaction with the Ni-NTA resin under native conditions. In the first case, most of the overproduced protein is found in the pellet of cellular debris after the first centrifugation step following cell lysis. In the second case, the overproduced protein remains in the supernatant after the first centrifugation step but is found primarily in the flow-through (FT) and wash fractions after application to the Ni-NTA agarose column. Proteins that form inclusion bodies are solubilized from the pellet of cell debris in 5 ml binding buffer at room temperature. Cell debris is recentrifuged at 15,000 g for 20 min at room temperature. For soluble proteins whose His-tag is not available, the frozen cells are resuspended in 10 mL of binding buffer at room temperature. The suspension is centrifuged for 30 min at 15,000 g at 4°C. Steps 6-11 of the native purification protocol (section 3.2) are then applied.

5. Buffers for purification under denaturing conditions are as follows: Binding buffer: 100 mM sodium phosphate, 10 mM Tris-HCl pH 7.8, 6 M guanidine. Wash buffer: 100 mM sodium phosphate, 10 mM Tris-HCl pH 6.3, 6 M guanidine. Elution buffer: 100 mM sodium phosphate, 10 mM Tris-HCl pH 4.5, 6 M guanidine.
6. Instead of the 2XYT medium, the use of tryptone phosphate medium has been described to prevent the inclusion body formation: 2% bactotryptone, 0.2% Na<sub>2</sub>HPO<sub>4</sub>, 0.1% KH<sub>2</sub>PO<sub>4</sub>, 0.8% NaCl, 1.5 % yeast extract, 20% glucose [6].
7. To avoid the formation of inclusion bodies, it is also possible to induce the culture at a temperature of 16°C for 16 h.
8. In the absence of an FPLC system, home-made columns can be packed using commercial Ni-NTA (Qiagen) or Co-Talon resins (Clontech) in 1 mL syringes, plugged with a circular piece of Whatman 3MM paper. Flow-rate is controlled at 10 mL/h by a peristaltic pump and fractions are collected using a basic model fraction collector (e.g. Frac-200, GE Healthcare).
9. At higher concentrations, certain proteins tend to aggregate and precipitate. In this case, it is possible to dilute the protein immediately after elution by adding buffer to the fraction collection tubes.
10. Some RNases (e.g. RNase Z) are rapidly inactivated in the presence of imidazole. We therefore do not wait for the result of the SDS-PAGE analysis before dialyzing.
11. It is possible to knot a piece of tissue paper and place in reservoir to accelerate the destaining process by absorption of the remaining dye.

12. When preparing the mix, it is highly recommended to assemble the reaction components at room temperature, to avoid precipitation of the DNA template with the spermidine present in the reaction buffer. Add DNA template and T7 RNA polymerase last.
13. Use RNase and DNase free water and buffers.
14. For higher yields of short transcripts you can add a higher concentration of the template or incubate for 4-6 hr. This allows each RNA polymerase molecule to engage in more initiation events.
15. If fragment to be generated is greater than 300 nts, increase cold UTP to 120  $\mu$ M.
16. All centrifugation and vortex steps are performed at 4°C and tubes are otherwise kept on ice at all times.
17. Use the same MgCl<sub>2</sub> concentration (i.e. 3 mM or 10 mM) as for step 5 of protocol.
18. Gradients should be prepared in advance, e.g. while cultures are growing, and left equilibrate at 4°C for at least an hour.
19. To get “pure” ribosome precursors, pick only the fractions corresponding to the central part of the peak.
20. The integrity of the rRNAs can be checked by loading fractions onto a 1% agarose gel containing 0.04% ethidium bromide. Do not denature the sample before loading to avoid RNA degradation due to the presence of Mg<sup>2+</sup> in Buffer A. If ribosomes are in good condition, rRNA(s) should appear as a clean and non-smearly band.
21. All dilutions are made in the enzyme Dilution buffer.
22. As a control, it is important to have the catalytic mutant of the protein.
23. Some enzymes, such as RNase J1, can work at 4°C; it is important to start the incubations at the desired temperature very quickly after adding the enzyme.
24. We always do two negative controls, one with just the RNA and no incubation (C0), to show the initial state of the RNA, and one where the RNA has been incubated without enzyme for the same duration as the experimental samples, e.g. (C20), to check that the RNA stays intact over the incubation period. In the case of a kinetic study, this corresponds to the longest incubation time.
25. 20% gels polymerize very fast. Polymerization can be slowed by reducing the amount of APS by half and keeping the mixture on ice.
26. Do not polymerize longer than 20 min to be able to remove the comb.
27. When the acrylamide concentration is higher than 10%, the gel doesn't stick well to Whatman 3MM paper. In this case, we apply Saran Wrap<sup>TM</sup> to the gel surface first. We

then peel off the gel attached to the Saran Wrap<sup>TM</sup> and then apply Whatman 3MM paper to the gel for drying.

28. If the substrate studied is not radiolabeled, once the migration is over, transfer the gel to a nitrocellulose membrane Amersham Hybond<sup>TM</sup>-N<sup>+</sup>. Probe the RNA with a specific radiolabeled probe, standard Northern Blot protocol, see Chapter X, Section 2.3 for details.

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### **Figure Legends**

**Figure 1.** Schematic representation of an RNase J1 5' exoribonuclease dose response assay measuring release of  $^{32}\text{P}$ -NMP from the 5' end of a radiolabeled 20 nt substrate in the presence of a 10-fold serial dilution of the enzyme. The higher concentration, the stronger the activity observed. The activity is 100 times weaker in a catalytic mutant.

**Figure 2.** Schematic representation of an RNase J1 5' exoribonuclease kinetic assay.  $^{32}\text{P}$ -NMP is released after 2 min and reaches a plateau at 10 min.