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## Chapter X

### **Analysis of *Bacillus subtilis* ribonuclease activity *in vivo*.**

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**Running head:** In vivo assays of *B. subtilis* RNases

## Summary

Ribonucleases remodel RNAs to render them functional or to send them on their way towards degradation. In our laboratory, we study these pathways in detail using a plethora of different techniques. These can range from the isolation of RNAs in various RNase mutants to determine their implication in maturation or decay pathways by Northern blot, to proving their direct roles in RNA cleavage reactions using purified enzymes and transcribed substrates *in vitro*. In this chapter, we provide in-depth protocols for the techniques we use daily in the laboratory to assay RNase activity *in vivo*, with detailed notes on how to get these methods to work optimally. This chapter complements chapter **Y** on assays of ribonuclease action *in vitro*.

**Keywords:** Ribonuclease, bacterial cell cultures, rifampicin kinetics, Northern blot, capillary transfer, electrotransfer, hybridization

## 1. Introduction

Ribonucleases (RNases) play key roles in remodelling RNA, by cutting it internally or progressively shortening it from either end (for review see [1]). This can have major consequences for gene expression. Cleaving an mRNA by an endoribonuclease within a coding sequence, for example, completely inactivates the RNA, rendering it useless for translation and typically sending the resulting RNA species on a pathway to full degradation. On the other hand, cleaving an mRNA within its 5' or 3' untranslated regions (UTRs), or in the intergenic regions between cistrons, can have either stabilising or destabilising effects, depending on the proximity of these cleavages to potential protective secondary structures, with a parallel effect on gene expression. Indeed, secondary structures at the extreme 5' or 3' end of RNAs typically protect them from degradation by exoribonucleases (and some 5'-end-dependent ribonucleases).

RNase cleavage of stable RNAs can also have a major impact on both their functionality and stability. The trimming of extraneous RNA from ribosomal RNA (rRNA) or transfer RNA (tRNA) precursors is a critical step in their becoming functional. Removal of 3' extensions from tRNAs precursors, for example, allows the universal CCA motif to become available for aminoacylation. Similarly, removal of the 3' extension of 16S rRNA improves the availability of the anti-Shine-Dalgarno (SD) sequence to bind to the SD sequence of the ribosome binding site of bacterial mRNAs. Failure to correctly process these RNAs, not only renders them less or non-functional, but also marks them for degradation by 5' and 3' exoribonucleases that use the extensions as landing pads to begin the decay process [2]. This is a recurring theme in quality control degradation pathways for non-functional stable RNAs.

Lastly, processing of small regulatory RNAs (sRNAs) can, like mRNAs and stable RNAs, influence both their functionality and stability. We have recently shown, for example, that the bacterial RoxS sRNA interacts better with its target mRNA *sucCD* when cleaved by

an endoribonuclease at position 20 [3]. This processing event is not necessary for interactions with other targets such as the *ppnKB* mRNA. Intriguingly, cleavage at this position is also the first step in the degradation of this sRNA. Thus, it appears that this degradation intermediate can be at least temporarily side-tracked from its ultimate fate to perform a specific regulatory function.

Understanding the roles of RNases in the maturation and degradation of mRNAs, stable RNAs or sRNAs requires measurement of half-lives or observation of differences in the sizes of RNA species in wild-type (wt) vs. mutant strains by Northern blot. In this chapter, we describe our current laboratory protocols for measuring mRNA half-lives in the Gram-positive model *B. subtilis*. In the accompanying chapter [Y](#), we describe how to assay RNase activity *in vitro*.

## 2. Materials

### 2.1 RNA decay experiments

1. Incubator.
2. Erlenmeyer flasks.
3. Benchtop Microcentrifuge.
4. Dry ice and dry ice bucket.
5. 15 mg/mL Rifampicin in dimethylsulfoxide (DMSO) or dimethylformamide (DMF)
6. 10 mM sodium azide in sterile, deionized water.
7. 10X TE/NaCl buffer: 100 mM Tris-HCl, pH 8, 10 mM EDTA and 1 M NaCl.
8. 10X phosphate citrate (PC) buffer: 107 g/L of anhydrous  $K_2HPO_4$ , 60 g/L of  $KH_2PO_4$  and 10 g/L of trisodium citrate pentahydrate, adjusted to pH 7 with KOH.
9. Growth medium, e.g. commercial rich medium (2xYT or LB), or minimal medium. For 25 mL of minimal MD medium, mix 2.5 mL 10X PC buffer, 0.125 mL of 2.2 mg/mL

ferric ammonium citrate CAF, 0.075 mL of 1M MgSO<sub>4</sub> and 1.25 mL of 40 mg/mL potassium aspartate (adjusted to pH 7 with KOH), 2% (w/v) carbon source (e.g. glucose or malate), and 0.25 mL of 5 mg/mL L-tryptophan for *B. subtilis* Trp auxotrophs.

10. *B. subtilis* strain of interest (our laboratory strain is the W168 strain SSB1002 [4]).

## ***2.2 Isolation of RNA from small culture volumes***

All materials and solutions must be RNase free and gloves should be worn during the whole procedure.

1. Benchtop Microcentrifuge.
2. 1.5 mL sterile microtubes.
3. Genie Digital Cell Disruptor (Thermo-Fisher Scientific).
4. Bucket containing dry ice.
5. UV-vis spectrophotometer, preferably with microvolume capability (e.g. Nanodrop instrument from ThermoFisher Scientific).
6. Vacuum drier.
7. Chemical fume hood.
8. Thermostated dry temperature block.
9. TE buffer: 10 mM Tris-HCl pH 8, 1 mM EDTA. Store on ice before use.
10. Acidic Phenol (water saturated solution, pH 5).
11. Chloroform.
12. 20% (w/v) Sodium dodecyl sulfate (SDS) solution.
13. 95% Ethanol.
14. 80% Ethanol stored at -20 °C.
15. Sterile (RNase free) deionized water. Keep an aliquot on ice during extraction with phenol.

16. 10 M LiCl in sterile, deionized water.
17. Acid-washed glass beads (150-220  $\mu\text{m}$  diameter).
18. RNA extraction solution (for RNAsnap<sup>TM</sup>): 95 % (v/v) Formamide, 18 mM EDTA, 0.025% (w/v) SDS, 1% (v/v) 2-mercaptoethanol. Prepare right before use (150  $\mu\text{L}$  per sample).

### ***2.3 Northern Blotting***

All tubes and solutions must be RNase-free.

#### ***2.3.1 General***

1. Water bath and thermostated dry temperature block.
2. Power supply for gel electrophoresis or electrotransfer.
3. Commercial high range or low range RNA markers for agarose and polyacrylamide gels, respectively.
4. Hybridization oven with a rotating rack for cylindrical glass hybridization bottles.
5. UV lamp (254 nm) for cross-linking. The UVP HL-2000 Hybrilinker (UVP) has both oven and cross-linker functions.
6. Benchtop microcentrifuge.
7. Geiger counter.
8. Pencil.
9. PhosphorImager (Typhoon, GE Healthcare) with dedicated <sup>32</sup>P phosphorimaging screens and image analysis software.
10. 10X Tris-Borate-EDTA (TBE) buffer. Buy RNase-free stock from reliable commercial source.
11. 1X TBE running buffer: Prepare 1L solution by dilution of 100 ml of 10X TBE stock with 900 ml of RNase-free water.

12. Gel Loading buffer: 95% (v/v) formamide, 18 mM EDTA and 0.025% (w/v) each of SDS, Xylene Cyanol, and Bromophenol Blue.
13. Nylon membrane (Hybond N+, GE Healthcare), same size as the gel.
14. Sheets of Whatman 3MM blotting paper (GE Healthcare), cut to the size of the agarose/polyacrylamide gel.
15. 20X saline-sodium citrate (SSC) buffer. 3 M sodium chloride, 300 mM trisodium citrate. Adjust to pH 7.0 with HCl.
16. Washing solution 1: Dilute the 20X SSC buffer to a final concentration of 2X SSC and add SDS to a final concentration of 0.1% (w/v).
17. Washing solution 2: Dilute the 20X SSC buffer to a final concentration of 0.2X SSC and add SDS to a final concentration of 0.1% (w/v).

### **2.3.2 Agarose gels (1%)**

1. 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).
2. High-grade agarose.
3. UV table and camera or dedicated gel imaging system.
4. Paper towels.
5. Plastic container.
6. Transfer buffer (5X SSC, 0.01M NaOH): Dilute the 20X SCC stock buffer to 5X with RNase-free water and add NaOH to a final concentration of 10 mM.
7. 1% (w/v) ethidium bromide in water. Handle reagent, solutions, waste, etc. with great care, following adequate CMR safety procedures.
8. Ultrahyb hybridization buffer (Invitrogen) for agarose Northern blots.



### **2.3.3 Polyacrylamide gels (5%)**

1. Gel electrophoresis system (OmniBLOT, Cleaver scientific, or equivalent) with 20x20 cm glass plates, 1 mm spacers and 24-well gel comb for polyacrylamide gel electrophoresis.
2. Trans-Blot cell (BioRad), or equivalent, for polyacrylamide gel transfer.
3. 5% acrylamide solution: For 1 L, mix 420 g of urea with 100 mL of 10X TBE buffer and 125 mL of 40% acrylamide:bis-acrylamide solution (19:1). Complete to 1 L with sterile, deionized water. Put the solution on a magnetic stirrer for at least 1 hour, until the urea is completely dissolved. Filter the solution with a 0.2  $\mu\text{m}$  pore-size filter. The solution can be stored at 4°C for several months in a dark bottle protected from light.
4. Silanization solution to pre-treat front gel plate (Sigma).
5. 10% (w/v) ammonium persulfate (APS) in water. Make 1 ml aliquots and store at -20 °C.
6. N,N,N',N'-Tetramethylethylenediamine (TEMED).
7. 0.5X TBE buffer, for electrophoretic transfer buffer. Prepare 4 L solution by dilution of 200 ml of 10x TBE buffer in 3.8 L of RNase-free water. Store at 4°C.
8. Roti-Hybri-Quick (Roth) for acrylamide Northern blots. Denature 20 mL of the 20 mg/mL stock solution herring or salmon sperm DNA and add to 1L Roti-Hybri-Quick. Store at 4°C in 100 mL aliquots.

### **2.3.4 Radiolabeling of oligonucleotide probe**

1. 10 mM Oligodeoxyribonucleotide complementary to mRNA (25 to 30-mer) in deionized water.
2. 10U/ $\mu\text{L}$  T4 Polynucleotide kinase (PNK) with supplied 10X PNK reaction buffer.
3. 3000 Ci/mmol  $\gamma^{32}\text{P}$ -ATP.
4. G50 Microspin column (GE Healthcare).

5. Screens and protective clothing to work with  $^{32}\text{P}$ .

### 3. Methods

#### 3.1. RNA decay experiments

Rifampicin is a broad-spectrum antibiotic used principally to treat tuberculosis. It inhibits initiation of transcription by binding the  $\beta$ -subunit of DNA dependent RNA polymerase (RNAP), allowing us to measure the rate of decay of RNA as a function of time, without interference from new transcription. If the stability of an mRNA is unknown, we typically isolate total RNA at several short and long time points (e.g. 2, 4, 6, 10, 20, and 30 min) after the addition of rifampicin to growing cultures in Erlenmeyer flasks. This can be adjusted to more appropriate time points once an initial decay pattern has been revealed. For practical reasons (i.e. it takes several seconds for rifampicin to mix evenly into the culture), our zero time point 'T0' actually corresponds to a sample taken 1 minute before the addition of rifampicin, with an assumption that relative amount of the mRNA of interest to all others will not significantly evolve in steady state growth during that minute (i.e. that T-1 = T0). The RNAs are probed by Northern blot (see below) for the specific mRNA of interest. The half-life ( $T_{1/2}$ ) of specific mRNAs is calculated by plotting the log of the percent remaining mRNA against time, with the initial amount of RNA 'T0' set at 100%. The slope of a linear regression of the data points on this graph is then used to calculate the half-life using the following formula:

$$T_{1/2} = \log 2 / \text{-slope}$$

An example is shown in **Figure 1**. [\[Figure 1 near here\]](#) Note that for some mRNAs, the decay curve will be biphasic rather than linear, usually signifying two conformations/populations of the RNA in the cell. Thus, a judgement needs to be made for which data points it is more pertinent to measure the half-life. For stable mRNAs, measurement of half-life is not useful,

as it usually surpasses the doubling time of the strain. However, a rifampicin kinetics experiment can be useful to follow the evolution of precursor species or decay products.

1. Prepare an appropriate number of 1.5 ml microtubes (1 for each time point) containing 100  $\mu$ l of 10 mM sodium azide and freeze at  $-20^{\circ}\text{C}$ .
2. Inoculate 5 ml of the specific medium of interest (e.g. 2xYT, LB or MD) with the *B. subtilis* strain of interest. Grow overnight at  $37^{\circ}\text{C}$  under shaking (200 rpm).
3. Use the overnight preculture to inoculate 15 mL of same medium culture (which is enough to measure the optical density at 600 nm [ $\text{OD}_{600}$ ] regularly and to harvest for each data point) in 150 mL Erlenmeyer flasks at an  $\text{OD}_{600}$  of 0.05. Incubate at  $37^{\circ}\text{C}$  with shaking at 200 rpm and regularly check  $\text{OD}_{600}$  (see **Note 1**).
4. As the  $\text{OD}_{600}$  approaches the required value (e.g. mid-log  $\approx 0.6$  for *B. subtilis*), put the Microtubes containing frozen sodium azide on dry ice except for the 'T0' tube, which is put directly on ice. At T-1, pipette a 1-1.4 mL aliquot of culture on top of frozen sodium azide. Return flask immediately to orbital shaker at  $37^{\circ}\text{C}$ . Invert tube/vortex the sample tube until sodium azide has thawed, killing bacterial metabolism and reducing the temperature in one step (see **Note 2**).
5. 1 minute later, add rifampicin to a final concentration of 150  $\mu\text{g/ml}$  (see **Note 3**) and immediately return flask to shaker.
6. Finish treating the 'T0' sample by centrifuging 1 minute at 16,000 g at  $4^{\circ}\text{C}$ .
7. Remove supernatant and put the pellet immediately in dry ice (see **Note 4**).
8. Repeat steps 6 and 7 for each time point after addition of rifampicin (T1, T2, T3, etc.) transferring the next azide-containing Microtube in the series from dry ice to ice just before harvesting (see **Note 2**).

### ***3.2 Isolation of RNA from small culture volumes.***

The following two protocols have been optimized to extract RNA from small culture volumes (1 - 4 mL). These procedures are the same whether used on bacterial pellets harvested during a rifampicin kinetics experiment or from untreated cultures, e.g to compare RNA profiles in bacterial strains harvested at the same OD or between wt and mutant strains. Phenol extraction of RNAs from cells is relatively slow (~4 h), but the samples are pure and can be used for both Northern blots (below) and enzymatic reactions, such as primer extension analysis. The RNAsnap<sup>TM</sup> procedure [5] is quick (~30 minutes) but the presence of 95% formamide in the final sample means that they can only be used for Northern blotting. Note that the presence of formamide in the sample also affects the migration of RNA samples in the 200-300 nt range on 5% polyacrylamide gels, altering migration position and significantly reducing band sharpness.

1. For extraction of RNA, harvest up to 4 x 1 mL bacteria cells in 1.5 mL microtubes, depending on anticipated abundance of the mRNA in question. Centrifuge 2 min at 16,000 g at 4°C.
2. Resuspend each 1 mL pellet in 100 µL ice cold TE buffer (see **Note 5**). If several pellets have been harvested, pool samples and re-centrifuge for 2 min at 16,000 g at 4°C. Remove the supernatant and keep pellets in dry ice or store at -20°C until RNA extraction.
3. Proceed to step 4 for extraction by the RNAsnap<sup>TM</sup> procedure, or to step 12 for extraction by phenol. A maximum of 12 samples can be extracted at a time with the Genie Cell Disruptor
4. Fill empty 1.5 mL microtubes to 100 µL mark with glass beads (one tube per sample).

- Resuspend frozen pellet from step 2 by vortexing in 150  $\mu\text{L}$  of RNA extraction solution and add immediately to tube with glass beads.
- Put the tube *immediately* on Genie Cell Disruptor and vortex at maximal speed.
  - Repeat steps 4-5 for each sample in sequence.
  - After the last tube has been added to the vortex, let all tubes vortex for a further 10 min.
  - Incubate tubes for 10 min at 80  $^{\circ}\text{C}$  in a thermostated dry temperature block. Then, centrifuge tubes at 16,000 g for 10 min at 4 $^{\circ}\text{C}$ .
  - Transfer 100  $\mu\text{L}$  of the supernatant to a clean microtube, avoiding pipetting the beads.
  - Determine RNA concentration from UV absorbance at 260 nm using an extinction coefficient of 0.025 ( $\mu\text{g}/\text{ml}$ ) $^{-1}$   $\text{cm}^{-1}$  and an equivalent dilution of the RNA extraction solution as blank (see **Notes 6, 7**).
  - Proceed to step 29.
  - Before starting phenol extraction, prepare the 4 following 1.5 mL microtubes per sample (see **Note 8**) and keep on ice:
    - Tube 1: Fill to 100  $\mu\text{L}$  mark with glass beads. Then, add 150  $\mu\text{L}$  acidic phenol, 25  $\mu\text{L}$  chloroform, and 6.25  $\mu\text{L}$  20% SDS in that order;
    - Tube 2: 200  $\mu\text{L}$  (1 vol) acidic phenol;
    - Tube 3: 200  $\mu\text{L}$  (1 vol) acidic phenol and 100  $\mu\text{L}$  (0.5 vol) chloroform;
    - Tube 4: 600  $\mu\text{L}$  (3 vol) 95% ethanol and 20  $\mu\text{L}$  (0.1 vol) 10 M LiCl.
  - Resuspend the frozen pellet from step 2 in 200  $\mu\text{L}$  ice cold TE buffer.
  - Transfer the 200  $\mu\text{L}$  pellet resuspension to Tube 1 and keep on ice.
  - Repeat steps 13 and 14 for each sample. Then, vortex all tubes for 3 min at maximum speed on the Genie Cell Disruptor. Centrifuge at 16,000 g for 10 min at 4 $^{\circ}\text{C}$ .
  - Transfer the upper phase under a fume hood to Tube 2 on ice. Vortex 2 min at maximum speed on the Genie Cell Disruptor.

17. Transfer the upper phase under a fume hood to Tube 3 on ice. Vortex 1 min at maximum speed on the Genie Cell Disruptor. Centrifuge at 16,000 g for 10 min at 4°C.
18. To precipitate RNAs, transfer the upper phase under a fume hood to Tube 4. Vortex 30 s on the Genie Cell Disruptor. Precipitate RNAs by incubating samples at -20°C overnight, or at -80°C for 30 min.
19. Centrifuge at 16,000 g for 30 min at 4°C.
20. Carefully remove the supernatant without disturbing the RNA pellet.
21. Add 200 µL cold 80% Ethanol to wash pellet. DO NOT VORTEX. Mix gently by inverting the tube 4 times. Centrifuge at 16,000 g for 5 min at 4°C.
22. Carefully remove the supernatant without disturbing the RNA pellet.
23. Add 200 µL cold 95% ethanol. DO NOT VORTEX. Mix gently by inverting the tube 4 times. Centrifuge at 16,000 g for 5 min at 4°C.
24. Carefully remove the supernatant without disturbing the RNA pellet.
25. Spin down any remaining drops of ethanol. Carefully remove as much of the remaining ethanol as possible without disturbing the RNA pellet.
26. Dry under vacuum no longer than 1 min (see **Note 9**).
27. Resuspend the RNA pellet in 50 µL ice-cold, RNase-free water. Vortex 1 min every 15 min and leave on ice, until dissolved (1h minimum).
28. Determine RNA concentration from UV absorbance at 260 nm using an extinction coefficient of  $0.025 (\mu\text{g/ml})^{-1} \text{cm}^{-1}$  and water as a blank.
29. Store tubes at -20°C.
30. Thaw the tubes on ice before aliquoting samples for Northern blotting.

### ***3.3 Northern Blot***

The Northern blot technique allows the detection of a specific RNA species among total RNA run on gels and is used both to quantify the amounts of the RNA of interest in a given (environmental, mutant) condition, and to determine the number and sizes of the different RNA species transcribed from a particular locus. This method therefore has major advantages over other techniques for quantifying RNA, such as qRT-PCR, in particular because the experimenter can immediately assess the quality of the RNA preparation and tell whether it is degraded or not. Depending on the anticipated size of the RNA being studied, total RNA is separated on agarose gels (typically 1% agarose for the detection of RNAs >300 nucleotides [nts]) or on polyacrylamide gels (typically 5% acrylamide for RNA molecules <300 nts). RNAs are transferred to nylon membranes by capillarity or electrophoretically, respectively. We describe here the two types of Northern blot (agarose and polyacrylamide) to detect RNAs with radioactive probes, which are typically an order of magnitude more sensitive than fluorescently labelled probes, for example.

### ***3.3.1 Gel electrophoresis and membrane transfer***

1. For detection of RNAs > 300 nts, proceed to step 2 for agarose gels. For longer RNAs, proceed to step 12 for polyacrylamide gels.
2. Prepare a 1% agarose gel by weighing 1 g of agarose and transferring to an Erlenmeyer flask. Add 100 mL of 1X TBE. Bring to the boil in a microwave. Remove from microwave, mix by swirling, bring back to boil 2-3 times until all agarose is solubilized. Cool solution until temperature is between 40° and 50°C. After cooling, add 6 µL of 1% ethidium bromide solution and pour the gel in a dedicated mold. Add the gel comb and allow gel to set for 1 hour.
3. Mix 5 µg of total RNA with an equal volume of gel loading buffer. The final volume should not exceed 20 µL.

4. Denature RNAs by heating samples for 5 min at 90°C. Then, load samples in gel wells.
5. Run the gel at room temperature at 100 V (Do not exceed 150 mA). Stop the migration when the bromophenol blue dye reaches the end of the gel (about 1 cm before the end of the gel).
6. Visualize the agarose gel on a UV table to check the integrity of ribosomal RNAs. Take photo to use as evidence of equal loading.
7. Set up the transfer in plastic tray by adding approximately 10 cm of paper towel (**Figure 2**). [Figure 2 near here]
8. Add 10 sheets of Whatman 3MM paper presoaked with transfer buffer. Then, add the Hybond N+ membrane presoaked with transfer buffer (**Figure 2**).
9. Place the gel on the membrane avoiding air bubbles between the gel and the membrane (**Figure 2**).
10. Add 1 sheet of Whatman 3MM paper (approximately 4 times longer than the height of the gel) to allow the paper to soak in the transfer buffer on both sides (**Figure 2**).
11. Place gel casting mold on top to stabilize the stack.
12. Transfer for at least 4 hours. (see **Note 10**). Then, proceed to section 3.3.2.
13. For vertical polyacrylamide gel electrophoresis, treat the notched glass plate with a few milliliters of silanization solution and spread evenly with a paper towel under a fume hood (see **Note 11**).
14. Mix 40 mL of the 5% polyacrylamide gel solution with 400 µL 10% APS and 40 µL TEMED. Pour gel in a preassembled 20 cm × 20 cm × 1 mm gel cassette and insert a 24-well gel comb immediately, without introducing air bubbles. Allow the gel to polymerize for 1 h at room temperature.
15. Mount gel on the electrophoresis apparatus and fill reservoirs with 1X TBE buffer.



16. Rinse the gel wells with a Pasteur pipette filled with 1X TBE to remove urea and polyacrylamide fragments.
17. Mix 5  $\mu\text{g}$  of total RNA with an equal volume of gel loading buffer. The final volume should not exceed 20  $\mu\text{L}$ .
18. Denature RNAs by heating samples for 5 min at 90°C. Rinse the gel wells rapidly again with a Pasteur pipette and then load RNA samples in the wells.
19. Run the gel at room temperature at 400V. Stop the migration when the bromophenol blue dye reaches about 1 cm from the bottom of the gel.
20. Separate the gel plates with the help of a spatula or a similar tool (see **Note 12**).
21. On the negative grid of the transfer cassette, add a sponge soaked in 0.5X TBE buffer.
22. Soak a piece a 3MM Whatman paper equal to the size of the gel (20 cm x 20 cm) in 0.5X TBE and place it on the sponge.
23. Soak a second 3MM Whatman paper equal to the size of the gel (20 cm x 20 cm) in 0.5X TBE and apply to the gel still attached to the glass plate. Optimize contact with the gel and remove excess liquid by blotting with a paper towel (see **Note 13**).
24. Peel the paper with the gel stuck to it from the glass plate and place it (gel facing up) on the first piece of Whatman 3MM paper.
25. Soak the Hybond N+ membrane equal to the size of the gel in 0.5X TBE and place it on the gel. Use a pipette or glass rod to roll out air bubbles.
26. Add two more sheets of 3MM Whatman paper pre-soaked in 0.5X TBE on the membrane, rolling out air bubbles.
27. Add a second sponge soaked in 0.5X TBE and close the transfer cassette.
28. Place the cassette in the BioRad Trans-Blot in the correct orientation (membrane towards positive pole) and fill the tank with 0.5X TBE precooled at 4°C.
29. Transfer for 2 hours at 250 mA and an additional 2 hours at 350 mA (see **Note 10**).

### ***3.3.2 Crosslinking and membrane pre-hybridization***

1. Disassemble the transfer system and dry the membrane on a sheet of 3MM Whatman paper.
2. Add a pencil mark to the RNA side of the membrane.
3. To cross-link the RNAs, place the membrane in the UV crosslinker with the RNA side facing upwards. Expose it to short-wave UV light (254 nm), at a dose of 120,000 mJ/cm<sup>2</sup>.
4. Place the membrane in cylindrical glass hybridization bottle.
5. Add 10 mL of prehybridization buffer (Ultrahyb for agarose and Roti-Hybri-Quick for acrylamide Northern). Pre-hybridize the membrane with rotation for at least 1h at 42°C (see **Note 14**).
6. While the membrane is pre-hybridizing prepare the labelled oligonucleotide probe (section 3.3.3)

### ***3.3.3 Oligonucleotide probe labelling and hybridization (see Notes 14, 15)***

1. Mix 1.5 µL of 10 mM oligonucleotide stock with 5 µL H<sub>2</sub>O, 1 µL of 10X PNK buffer, 1 µL of T4 PNK and 1.5 µL of  $\gamma^{32}\text{P}$ -ATP. Incubate at 37 °C for 45 min.
2. Prepare the G50 Microspin column by inverting the column several times to homogenize the contents and centrifuge 1 min at 800 g to remove the storage buffer.
3. Add 40 µL H<sub>2</sub>O to the labelling reaction, vortex to mix and apply to the G50 Microspin column.
4. Centrifuge 2 min at 800 g and recover flow-through (FT). Check labelling efficiency is >50% by comparing radioactivity in FT solution (incorporated) versus that retained on column (free) using Geiger counter.

5. Add all of the radioactive probe (~50  $\mu$ L) to tube containing pre-hybridizing membrane (section 3.3.2) and hybridize with rotation for at least 4h at 42°C (see **Note 14**).

### ***3.3.4 Membrane washing and RNA detection***

1. Remove and safely discard the radioactive hybridization buffer (see **Note 15**).
2. Rinse the membrane briefly with at least 10 mL of washing solution 1 by shaking the tube and discard safely the washing buffer (see **Note 15**).
3. Add 10 mL of the washing solution 1, and place the tube in the hybridization oven for 10 min at 42°C. Then, discard the washing solution safely (see **Note 15**).
4. Add 10 mL of the washing solution 2 and place the tube in the hybridization oven for 10 min at 42°C. Then, discard the washing solution safely (see **Note 15**).
5. Repeat step 4 two more times.
6. Air-dry the membrane on a sheet of 3MM Whatman paper, cover both with plastic wrap and expose it to a PhosphorImager screen overnight (4 hours can be sufficient if the probed RNA is abundant).
7. Reveal image on a PhosphorImager. In a rifampicin experiment, typically we will see the band corresponding to the mRNA of interest decreasing steadily over time. Stable RNAs (e.g. ribosomal RNAs) will not decrease and indeed can be used to normalize for loading variations. RNA sizes may also change e.g. when analyzing mutant strains lacking processing enzymes.

## **4. Notes**

1. It is best to work directly in a 37°C room or right next to a 37°C incubator (air shaker or water bath).

2. For data points that are close together, it is advisable to find a lab partner to run the centrifuge, remove supernatants and freeze samples, while the other adds rifampicin, samples the cultures and mixes with frozen azide.
3. Rifampicin is light sensitive and stored resuspended in DMSO or DMF in foil covered tubes; wear gloves during manipulation.
4. For minimal medium (e.g. MD or M9), it is important to wash the pellet by resuspending in 1 mL of TE/NaCl buffer to remove excess phosphate and to centrifuge a second time before freezing the pellet on dry ice.
5. When working with RNAs be careful to avoid any contamination with RNases. Wear gloves at all times, and prior to your experiments clean your bench and your pipettes with water then ethanol or RNase away solution. We keep a separate set of micropipettes reserved for RNA work.
6.  $OD_{260}/OD_{280}$  ratios are generally poor for RNAsnap<sup>TM</sup> preps
7. Samples should be equilibrated at room temperature before measurement of RNA concentration to avoid interference from the SDS present in the solution that precipitates in the cold.
8. Work under a chemical fume hood.
9. Be careful not to dry the pellet too much because it will be difficult to resuspend.
10. After transfer, RNAs can be visualized with a portable UV light at 254 nm. Mark the ribosomal RNAs and the bands of the RNA marker with a pencil, pressing strongly. This will score the membrane and hold the radioactivity during the hybridization, allowing the visualization of the marker without its labelling.
11. Silanization of the plate should be done under a fume hood (only 1 plate should be treated!)
12. Use a plastic tool to avoid damaging the glass plates.

13. Dry the Whatman 3MM paper with paper towel to remove liquid excess and allow the gel to stick to the Whatman 3MM paper.
14. If the RNA of interest is expressed at a very low level, the radiolabeled oligonucleotide can be replaced by an  $\alpha$ -<sup>32</sup>P -UTP labelled riboprobe complementary to the RNA. This will often improve the signal, but note that it is difficult to remove if the membrane is to be reprobbed for another RNA. We usually make riboprobes between 200 and 500 nucleotides in length, using a PCR template with a T7 promoter integrated into the lower oligo (see chapter Y). The pre-hybridization and hybridization temperatures should be increase to 68°C if a riboprobe is used instead of radiolabeled oligonucleotide.
15. All radioactive materials should be handled by properly trained scientists, following safety rules and local regulations.

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## **FIGURE LEGENDS**

**Figure 1:** Example of half-life measurement.

**Figure 2:** Schematic of capillary transfer set up for agarose gels