Supporting Protocol: Purification of the *M. genitalium* RNase R enzyme

Kady-Ann Steen, Nathan A. Siegfried & Kevin M. Weeks

The *rnr* gene of *M. genitalium* G37 codes for RNase R; this coding region was previously adapted for expression in *E. coli* by Lalonde *et al.*[1]. Using this construct (a generous gift of the laboratory of Zhongwei Li), we placed the coding region in a pET-21d (EMD Biosciences) vector to add a C-terminal $(His)_6$ tag for affinity purification. This supporting protocol presents a streamlined purification for this affinity-tagged RNase R protein construct.

RNase R can be handled safely in an RNA lab if care is used to prevent cross-contamination of other experiments. RNase R purification should be carried out in an isolated lab area. Glassware and other items should be sequestered for "RNase use only." Bench paper should be used and disposed of immediately after use (as should all RNase-related waste). Reactions containing RNase R are readily inactivated by heating to 95 °C for 3 min in the presence of EDTA[2].

REAGENTS

- pET-21d(+) plasmid vector containing the *M. genitalium* RNase R coding sequence
- One Shot BL21(DE3) Chemically Competent *E. coli* (Invitrogen, cat. no. C6000-03)
- LB Broth, Miller (LB) (Fisher, cat. no. BP1426-500)
- LB Agar, Miller (Fisher, cat. no. BP1425-500)
- SOC Medium (Invitrogen, 15544-034)

- Isopropyl-β-D-Galactopyranoside, 1 M (IPTG) (Fisher, cat. no. BP-1620)
- Ampicillin, Sodium Salt, 100 mg/mL (Amp) (Fisher, cat. no. BP1760)
- Glycerol (Fisher, cat. no. BP229-1)
- β-mercaptoethanol (β-ME) (EMD, cat. no. 444203)
- Imidazole (Acros Organics, cat. no. 122025000)
- Dithiothreitol, 1 M (DTT) (Fisher, cat. no. BP172-25)
- NaCl (Fisher, cat. no. BP 3581)
- Ni^{2+}-NTA Resin (5Prime, cat. no. 24000)
- SDS-PAGE reagents (Invitrogen, Novex)

REAGENT SETUP

LB, autoclaved (3 L total required; 2 L in 4 L baffled flasks, 1 L in a glass bottle)

LB-Amp Plates, containing 100 µg/mL ampicillin

Lysis Buffer, Low Salt (50 mM Tris, pH 7.8, 300 mM NaCl, 5% glycerol), prepare 500 mL

Lysis Buffer, High Salt (50 mM Tris, pH 7.8, 700 mM NaCl, 5% glycerol), prepare 1 L

TE (10 mM Tris, 1 mM EDTA, pH 8.0)

Dialysis Buffer (40 mM Tris, pH 7.5, 250 mM NaCl, 2 mM DTT, 50% glycerol), DDT should be added immediately before use, prepare 4 L

Imidazole Buffer (3.4 g imidazole, pH to 8 with HCl, 2.5 mL of 1 M Tris, pH 7.8, 0.875 g NaCl, 3.13 mL 80% glycerol), prepare 50 mL, sterile filter

EQUIPMENT

- Sonicator
- SDS-Page apparatus (XCell *SureLock* MiniCell CE, Invitrogen)
- Stir plate
- Floor centrifuge (Sorvall, SS34 and GS3 rotors)
- Shaker
- Labquake tube rotator at 4 °C
- Tabletop centrifuge with 50 mL conical tube adaptors at 4 °C
- Sterile Petri dishes
- Autoclave
- Two 4-L autoclaved baffled flasks
- Autoclaved centrifuge bottles and tubes, 500 and 50 mL for SS34 and GS3 rotors
- 100 and 200 mL beakers
- 14-mL round bottom tubes (Thermo Scientific, cat. no. 150268)
- Float-A-Lyzer G2 dialysis tubes, 50,000 MWCO (Spectrum, G235058)
- Heat block

PROCEDURE

RNase R Expression Timing 2-3 days

1. Transform pET-21d plasmid vector into competent *E. coli* BL21(DE3) cells. If using

One Shot BL21 cells, add 1 μL plasmid to 1 tube of cells, mix gently, chill cells on ice for 5 min, heat shock at 42 °C for exactly 30 sec without mixing, then place cells on ice for 5 min. Add 250 μL room temperature SOC and shake at 37 °C for 1 hr to allow expression of antibiotic resistance gene.

2. Plate several dilutions of transformed cells on pre-warmed LB-Amp plates and incubate at 37 °C overnight.
3. The following morning remove plates from the incubator and store at 4 °C until evening to prevent overgrowth.
4. Inoculate two culture tubes containing 5 mL of LB with one colony each, add 5 μL 100 mg/mL ampicillin. Incubate in a shaker overnight at 250 rpm and 37 °C.
5. The following morning warm two 4-L baffled flasks containing 1 L of LB each to 37 °C. Inoculate each flask with a 5-mL overnight culture, add 1 mL of 100 mg/mL ampicillin and immediately take an optical density reading at 600 nm (OD_{600}).
6. Incubate flasks by shaking at 250 rpm at 37 °C. Monitor until OD_{600} is ~0.5. Remove 500-μL sample, mark "uninduced" and freeze. Add 1 mL of 1 M IPTG to each 1 L of culture to induce expression of RNase R. Reduce temperature to 25 °C.

CRITICAL: RNase R expression is significantly enhanced if overexpression is carried out at 25 °C rather than at 37 °C.

7. Continue incubation for 5 hrs. Remove 500-μL aliquot, label "induced" and store at -20 °C.
8. Pellet cells by spinning at 4,500 rpm (3,400 ×g) for 15 min in two 500-mL centrifuge bottles. Multiple spins will be required to pellet 2 L of cell culture in these two bottles. Following the first spin, discard the supernatant and refill the centrifuge bottle with cell

culture and spin again. At the end of this process, each bottle will contain a pellet corresponding to 1 L of LB culture.

PAUSE POINT. Cell pellets can be stored at -80 °C overnight.

Purification of RNase R Timing 2 days

1. Thaw frozen pellets on ice for 15 min.
2. Add 175 µL and 350 µL fresh β-ME to the low and high salt lysis buffers, respectively.
3. Resuspend each pellet in 6.25 mL low-salt lysis buffer.
4. Combine suspended pellets into one 50-mL glass beaker, place beaker into a larger beaker containing an ice and water bath and sonicate. Be careful not to heat the *E. coli* sample while sonicating. Cycle 15 seconds sonication and 30 seconds rest until sample viscosity and color are reduced.
5. Remove a 500-µL aliquot, label "cell lysate" and store at -20 °C.
6. Centrifuge for 30 min at 18,000 rpm (39,000 ×g) in a SS34 rotor (Thermo Scientific) to clear lysate, then pour supernatant into a clean centrifuge bottle and repeat.
7. During centrifugation, prepare the Ni^{2+}-NTA resin. Stir the resin into slurry immediately prior to pipetting, as it will quickly settle. Add 20 mL slurry to a 50-mL Falcon tube; spin at 1,500 rpm (350 ×g) for 1 min in a tabletop clinical centrifuge (at 4 °C). Remove and discard supernatant.
8. To exchange resin buffer, add 5 mL of low-salt lysis buffer to the Ni^{2+}-NTA resin, then place the tube in a tube rocker (also at 4 °C) and allow the resin to mix for 5 min. Centrifuge the tube at 1,500 rpm; remove and discard the supernatant. Repeat two more times.

9. Retrieve cleared lysate from the centrifuge, remove a 500 µL aliquot, label as "supernatant" and store at -20 °C. Apply the remaining supernatant to the prepared nickel resin and rock tube for 90 min.
10. Centrifuge the tube at 1,500 rpm for 1 min, remove and save supernatant, label as "flowthrough" and place on ice.
11. In a 300 mL beaker, combine 200 mL high salt lysis buffer and 4 mL of imidazole buffer. This combined solution (wash buffer, containing ~20 mM imidazole) will be used to wash the resin three times. Add 30 mL wash buffer to the resin and rock the tube for 10 min. Centrifuge at 1,500 rpm for 1 min, remove and save supernatant, label as "Wash 1" and place on ice.
12. As in Step 11, add 30 mL wash buffer to the resin, rock tube for 10 min, centrifuge at 1,500 rpm for 1 min, and remove and save supernatant as "Wash 2." Repeat for Wash 3.
13. In a 100-mL beaker combine 50 mL high salt lysis buffer and 20 mL imidazole buffer to make the elution buffer (containing 286 mM imidazole). Add 10 mL of this solution to resin and rock the tube for 30 min. Spin as above. Remove and save supernatant as "Elution 1."
14. Add another 10 mL of elution buffer to the resin as in Step 13. Repeat Step 13, save supernatant as "elution 2."
15. Pipette enzyme samples into Float-A-Lyzer G2 dialysis tubes and dialyze against 400 mL low salt lysis buffer for 2 hrs.
16. Prepare 4 L of dialysis buffer by adding DTT to a final concentration of 2 mM, then add 2 L of this buffer to a large beaker on a magnetic stir plate at 4 °C. Save a 1-mL aliquot of dialysis buffer to serve as a blank for quantifying enzyme concentration.

17. Transfer dialysis tubes to the beaker, dialyze for 8 hrs, replace dialysis buffer, dialyze overnight.
18. Quantify protein concentration using a sample of the final dialysis buffer as a blank when obtaining the protein absorbance. The extinction coefficient (at 280 nm) for the RNase R enzyme is 62,000 M^{-1} cm^{-1} (ref. 1). This procedure typically yields an enzyme solution of 5-12 μg/μL; however, it is important to make sure the enzyme stock concentration is at or above the experimentally useful concentration of 4.5 μg/μL[2].

SDS-PAGE Analysis Timing 90 min, overnight

1. Results of the RNase R purification procedure should be evaluated by analysis of the aliquots collected throughout this protocol by SDS-PAGE. Any appropriate gel electrophoresis system can be used. We typically use the Novex System (Invitrogen). Gel analysis should be carried out according to standard denaturing protein gel protocols (for example, see Ref. 3). Samples to be analyzed include uniduced cells, induced cells (from addition of IPTG), lysate, supernatant, washes 1, 2, and 3, and elutions 1 and 2.
2. To lyse uninduced and induced samples prior to analysis, first centrifuge at 10,000 g for 5 min, decant and discard supernatant.
3. Resuspend pellets in 75 μL of a gel loading buffer containing SDS, add 75 μL TE, then add 1.5 μL of β-ME (1% v/v final).
4. Heat the two samples at 90 °C for 5 min, vortex briefly, then centrifuge resuspended cells at 14,000 g for 10 min.
5. Prepare each of the other samples by combining 75 μL sample with 75 μL SDS gel loading buffer. Heat at 90 °C for 5 min, vortex, and spin briefly.
6. Load samples (~13 uL each) and a standard protein size ladder onto the gel, then run at

150 V for ~1 hr.

7. The protein content of each sample can be visualized by Coomassie or silver staining[3].
8. Dry gel for permanent record. A representative gel showing purification of the *M. genitalium* RNase R (75,000 Da) is shown in Fig. S1. Enzyme is typically ≥95% homogenous as judged by size and contains no significant non-RNase R activity.

REFERENCES

1. Lalonde, M.S. et al. Exoribonuclease R in Mycoplasma genitalium can carry out both RNA processing and degradative functions and is sensitive to RNA ribose methylation. *RNA* **13**, 1957-1968 (2007).

2. Steen, K.A., Malhotra, A. & Weeks, K.M. Selective 2'-hydroxyl acylation analyzed by protection from exoribonuclease. *J Am Chem Soc* **132**, 9940-9943 (2010).

3. Schagger, H. Tricine-SDS-PAGE. *Nat. Protocols* **1**, 16-22 (2006).

Figure S1. Representative SDS-PAGE analysis of RNase R purification. Total cell lysates prior to (–) or post (+) induction with IPTG are shown. Supernatant (S) and Washes 1, 2 and 3 contain proteins not retained on the Ni^{2+}-NTA resin. A large product band at the correct molecular mass (75 kDa) is present in the Elution (E1) lane. M, protein molecular mass standards (Novex). Gel lanes have been straightened in this image, but not otherwise altered.

Induction
Washes
M − + S 1 2 3 E1 M
160 kDa
80
60
40
20
10

Steen et al., Supporting Figure 1