Chapter xx

Purification of eukaryotic exoribonucleases following heterologous expression in bacteria and analysis of their biochemical properties by *in vitro* enzymatic assays

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Running head: Biochemical characterization of eukaryotic exoribonucleases

Abstract

Exoribonucleases – among the other RNases – play a crucial role in the regulation of different aspects of RNA metabolism in the eukaryotic cell. To fully understand the exact mechanism of activity exhibited by such enzymes, it is crucial to determine their detailed biochemical properties, notably their substrate specificity and optimal conditions for enzymatic action. One of the most significant features of exoribonucleases is the direction of degradation of RNA substrates, which can proceed either from 5'-end to 3'-end or in the opposite way. Here, we present methods allowing the efficient production and purification of eukaryotic exoribonucleases, the preparation and labeling of various RNA substrates, and the biochemical characterization of exonucleolytic activity. We also explain how the exonucleolytic activity may be distinguished from that of endonucleases.

Key words Exoribonuclease; RNase; RNA degradation; RNA; oligonucleotide labeling; isotope; fluorescent dye; enzymatic activity; thin layer chromatography; denaturing polyacrylamide gel.

1. Introduction

RNA metabolism in the eukaryotic cell is based on a wide range of enzymatic processes that might be considered mainly as RNA synthesis and degradation. During RNA maturation and decay, various steps of trimming and degradation are catalyzed by cellular enzymes called exoribonucleases ***(1, 2)***. RNA degradation may be initiated from the 5'-end or 3'-end and is dependent on the ability of the exoribonuclease to digest the substrate in a particular direction ***(1, 2)***. Exoribonucleases are thus either 3' to 5' (3'-5') or 5' to 3' (5'-3') degrading enzymes. Numerous representatives of each class are found in eukaryotic cells ***(1, 2)***.

The key eukaryotic 3'-5' exoribonucleases present both in the cytoplasm and the nucleus are proteins belonging to DIS3 family ***(3–6)***. Together with a 9-subunit ring-like scaffold regulating its nucleolytic activity, DIS3 forms a complex called the RNA exosome **(*6–10*)**. This RNase is responsible for degradation of properly synthetized transcripts as part of general RNA turnover following their deadenylation (removal of poly(A) tails and associated poly(A)-binding proteins from the 3'-end of RNA) but is also involved in quality control processes ensuring that various aberrant RNA molecules are eliminated from the cell ***(3)***. DIS3 also plays a role in RNA maturation by trimming precursors of different RNA classes to the proper length and also removes the RNA processing by-products ***(3)***.

DIS3 was initially identified as a hydrolytic 3'-5' exoribonuclease, dependent on manganese ions as a cofactor ***(11–13)***. Hydrolytic exonucleases utilize water molecules as nucleophiles when attacking the phosphodiester bond during digestion and produce nucleoside monophosphates as degradation products. The exoribonucleolytic activity of DIS3 was found to be strictly associated with a specific domain of the protein called RNB ***(13)***.

Recent studies carried out in independent laboratories demonstrated that DIS3 displays additional nucleolytic activity. Notably, another domain of DIS3, called PIN, supports endoribonucleolytic activity ***(14–16)***. In other words, the PIN domain of DIS3 is able to mediate phosphodiester bond cleavage directly at internal positions of RNA molecules. This endoribonucleolytic activity has requirements that differ significantly from those of the RNB-sustained exonuclease, notably with respect to the presence of manganese ions ***(14, 15)***. This single discovery demonstrates the importance of defining the exact manner by which a given ribonuclease triggers RNA degradation. It also underscores the necessity to optimize experimental parameters for each individual enzyme and RNA cleavage reaction.

The major eukaryotic exoribonuclease working in the direction opposite to DIS3 is XRN1 ***(17)***. This cytoplasmic 5'-3' hydrolytic RNase is involved in mRNA decay as well as in the degradation of RNA molecules arising from endonucleolytic cleavage events. A notable characteristic of XRN1 is its strong preference for RNA substrates with monophosphorylated 5'-ends. In other words, XRN1 is a 5'-3' exonuclease sensitive to the 5'-end phosphorylation status ***(18)***. This explains why removing the cap structure from mRNA 5'-ends (de-capping) is a prerequisite for XRN1 activity *in vivo* ***(19)***. *In vitro*, the presence of a triphosphate, hydroxyl group, or cap structure at the 5'-end of the RNA substrate prevents or at least significantly diminishes XRN1 activity ***(20)***.

To assess if a protein is an exoribonuclease, a simple assay based on incubation with an internally labeled RNA substrate (obtained by *in vitro* transcription in the presence of a radioactive NTP) and analysis of the products by thin layer chromatography (TLC) may be used. Release of NMP is then an indication of hydrolytic exonuclease activity ***(21****, see Fig. 1c therein****)***. However, to determine the directionality of RNA degradation by an

exoribonuclease and to distinguish its biochemical properties from those of endonucleolytic enzymes, more complex activity assays (with differentially labeled RNA oligonucleotide substrates) are required. In principle, three types of RNA substrates need to be probed in such assays: linear substrates with a radioactive or fluorescent label at the 5'- or 3'-end and circular RNA substrate (**Fig. 1**). *[Note to publisher: Figure 1 near here].*

The type of nuclease activity supported by a given protein is deduced from the nature of labeled RNA products obtained in the assays. If an enzyme generates nucleoside monophosphate from 5'-end labeled RNA as well as fragment(s) of several nucleotides (or a ladder of degradation products) from a 3'-end labeled substrate, then it is a 5'-3' exoribonuclease (**Fig. 1a**). A 3'-5' exoribonuclease should in turn generate nucleoside monophosphate from a 3'-end labeled RNA substrate and RNA fragment(s) (or ladder) from a 5'-end labeled substrate (**Fig. 1b**). A feature allowing to unambiguously discriminate between endo- and exoribonucleolytic activity is an inability of the latter (irrespective of the direction in which RNA degradation is carried out) to act on circular RNA substrates (**Fig. 1a,b**). By contrast, endoribonucleases can cleave and linearize circularized substrates (**Fig. 1c,d**). With end-labeled linear substrates, endoribonucleases will generate RNA fragments of defined lengths if they are using a specific cleavage site ("processing" endonucleases) (**Fig. 1c**) or ladders of decay products if they do not have clear substrate specificity ("degradative" endonucleases) (**Fig. 1d**). These various patterns of RNA degradation are usually determined upon analysis by denaturing polyacrylamide gel electrophoresis (PAGE) and by a gel imaging method compatible with the RNA labels (i.e., radioactive or fluorescent).

One characteristic of exoribonucleases is their degree of processivity, from purely processive to distributive enzymes. Distributive exonucleases release their RNA substrate

following each removal of a nucleotide from the molecule extremity and must rebind RNA prior to each new cycle of catalysis. Such nibbling activity is usually manifested on a PAGE gel by RNA degradation ladders (below the full-length RNA band) resulting from the formation of multiple digestion products, each differing from the next by only one nucleotide. By contrast, processive exonucleases digest RNA substrates in a continuous manner, without releasing them between consecutive rounds of catalysis. This is reflected by the fast appearance of the final, short degradation product in the time course of the reaction. In the extreme case of a highly-processive exonuclease and if there is no structural constraint within RNA (which may impair degradation), the only detectable products may be mononucleotides irrespective of whether 5'-end or 3'-end labeled RNA molecules are used (**Fig. 1a,b**). In this case, determination of directionality requires a RNA substrate containing an asymmetrically-positioned component able to inhibit the progression of the exoribonuclease (**Fig. 1e**) ***(22)***. Mononucleotides will be detected only if the label is present at one extremity of the RNA molecule while degradation of substrates labeled at the opposite end will give rise to the appearance of a detectable degradation intermediate (**Fig. 1e**) ***(22)***. The length of the intermediate is dependent on the site of modification and thus provides an unequivocal solution to the puzzle of whether the exoribonuclease digests RNA substrates in the 5'-3' or 3'-5' direction.

In vitro examination of the biochemical properties of a particular exoribonuclease is an essential part of the characterization of the enzyme and often helps in better understanding of the role played by the exonuclease of interest *in vivo*. The methods described herein provide a complete tutorial allowing the comprehensive investigation of the enzymatic activity of eukaryotic exoribonucleases, which was tested and proved to be successful in multiple cases in our laboratory. Although these methods are directly relevant

to the analysis of hydrolytic enzymes, they may be relatively easily adapted to phosphorolytic exonucleases, such as polyribonucleotide phosphorylase (PNPase), which utilizes orthophosphate instead of water during nucleophilic attack at the phosphodiester bond in RNA and produces nucleoside diphosphates as the reaction products. In such case, inorganic phosphate must be present in the reaction buffers.

We begin this chapter with methods for cloning an exonuclease coding sequence into a plasmid vector enabling the expression of the protein fused to 6xHis-SUMOTag at its N-terminus (section 3.1) and construction of its variant for expression of the catalytic mutant (section 3.2), which serves as an essential negative control in RNase activity assays. Next, we present methods for the production (section 3.3) and purification (section 3.4) of recombinant exonucleases using the 6xHis-SUMOTag. A preliminary activity assay employing uniformly labeled transcript and TLC analysis is then described (section 3.5). Subsequent sections are dedicated to the description of the methods allowing purification of RNA oligonucleotides (section 3.6) and preparation of substrates labeled in different ways (section 3.7). Finally, we describe the procedures for gel-based assays of exoribonucleolytic activity (section 3.8), including one allowing study of the impact of 5'-end phosphorylation status on 5'-3' exoribonuclease activity (section 3.9). Two main eukaryotic exoribonucleases mentioned earlier, namely DIS3 and XRN1, were used in the experiments illustrating the methods presented in this chapter.

2. Materials

2.1. Preparation of the expression vector coding for the exonuclease of interest with an N-terminal 6xHis-SUMOTag

1. A construct encompassing the exonuclease coding sequence (*see* **Note 1**), to be used as a template for amplification of the insert comprising the exonuclease open reading frame.
2. 200 µL PCR microtubes.
3. Phusion High Fidelity DNA Polymerase with 5x Phusion HF buffer and 100% DMSO (Thermo Scientific).
4. 10 mM dATP, dGTP, dCTP, and dTTP stocks or 10 mM dNTP mix (*see* **Note 2**). Store at -20°C as 50 µL aliquots.
5. SLIC-F (forward) primer: 5'-*TGATTGAAGTCTACCAGGAACAAACCGGT***ggatcc**ATGN_{17-22}-3'; nucleotides in italics are complementary to the pET28M-6xHis-SUMOTag vector; *Bam*HI site is bolded; underlined triplet represents translation initiation codon; N_{17-22} corresponds to the nucleotides complementary to the insert, directly following ATG triplet.
6. SLIC-R (reverse) primer: 5'-*CGGATCTCAGTGGTGGTGGTGGTGGTG***ctcgag**TTAX_{17-22}-3'; nucleotides in italics are complementary to the pET28M-6xHis-SUMOTag vector; *Xho*I site is bolded; underlined triplet represents translation termination codon; X_{17-22} corresponds to the nucleotides complementary to the insert, directly preceding STOP codon and should be in reverse complement.
7. Sterile water.
8. Thermocycler.
9. 1% agarose gels containing 1x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) or 1x TBE (89 mM Tris-borate, 1 mM EDTA, pH 8.3).
10. 40000x Ethidium bromide stock at 10 mg/mL in water.
11. DNA molecular weight marker.
12. UV transilluminator coupled with a camera.

13. Refrigerated centrifuge.
14. (25/24/1) Phenol/chloroform/isoamyl alcohol (v/v/v) mix (*see* **Note 3**).
15. Chloroform.
16. 3 M sodium acetate (NaOAc), pH 5.2.
17. 96% (v/v) and 75% (v/v) ethanol.
18. 6x Orange G loading solution: 10 mM Tris-HCl pH 7.6, 0.15% (w/v) Orange G, 60% (v/v) glycerol, 60 mM EDTA.
19. 10000x GelGreen™ (Biotium) stock in water.
20. UV/vis transilluminator (e.g., Dark Reader blue light transilluminator from Clare Chemical Research).
21. Sterile scalpel blades.
22. Commercial gel extraction kit (e.g., QIAquick Gel Extraction Kit from Qiagen).
23. Thermoblock.
24. pET28M-6xHis-SUMOTag plasmid vector.
25. *Bam*HI and *Xho*I restriction endonucleases with supplied buffer.
26. 20 mg/mL BSA.
27. 3 U/µL T4 DNA polymerase.
28. Chemo-competent *E. coli* MH1 strain (*araD lacX74 galU hsdR hsdM rpsL*).
29. SOB (Super Optimal Broth) liquid medium: 2% bacto-tryptone, 0.5% bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$.
30. LB-agar plates containing 50 µg/mL of kanamycin.
31. Standard LB growth medium supplemented with 50 µg/mL of kanamycin.
32. Incubation shaker.
33. Commercial, column-based kit for small-scale plasmid purification from bacterial cells.

34. Set of primers for sequencing of the insert cloned into pET28-6xHis-SUMOTag (unique for a given insert).

2.2 Construction of the expression vector encoding the exonuclease with an inactivating mutation in the catalytic site

1. MUT-F (forward) and MUT-R (reverse) primers to be used during site-directed mutagenesis (*see* **Note 4**).
2. Sterile water.
3. F-dye (formamide loading dye): 90% deionized formamide in 1x TBE buffer, 0.03% (w/v) xylene cyanol, 0.03% (w/v) bromophenol blue, 20 mM EDTA.
4. Thermoblock.
5. 20% denaturing acrylamide stock: 20 % acrylamide:bisacrylamide (19:1) and 8 M urea in 1x TBE buffer. Filter with a 0.2 µm (pore size) filter unit and store at 20^{o}C in a glass bottle wrapped with aluminum foil to protect from light (*see* **Note 5**).
6. "0% acrylamide" solution: 8 M urea in 1x TBE. Filter with a 0.2 µm (pore size) filter unit.
7. 10% denaturing polyacrylamide gel (20 cm x 20 cm): a short time before use, mix 25 mL of 20% denaturing polyacrylamide stock with 25 mL of "0% acrylamide" solution, 300 µL of 10% ammonium persulfate (APS), and 30 µL of N,N,N',N'-tetramethylethylenediamine (TEMED) (*see* **Note 6**). Pour mixture into gel plates equipped with 1 mm-thick spacers, make sure to avoid air bubbles, and insert the appropriate comb (*see* **Notes 7** and **8**). Wait for approximately 30 minutes until polymerization is complete.
8. Vertical gel electrophoresis system with power supply.
9. Saran film.
10. Hand-held UV lamp.

11. Sterile scalpel blades.

12. Laboratory burner or lighter.

13. Refrigerated centrifuge.

14. Liquid nitrogen (in a styrofoam box or in a thermos).

15. Forceps.

16. DNA elution buffer: 1 M ammonium acetate, 1 mM EDTA, 0.1% SDS.

17. 2-propanol.

18. Microvolume spectrophotometer (e.g., NanoDrop 2000 from Thermo Scientific).

19. Phusion High Fidelity DNA Polymerase with 5x Phusion HF buffer and 100% DMSO (Thermo Scientific).

20. 10 mM dNTP Mix (*see* **Note 2**).

21. Thermocycler.

22. 1% agarose gels containing 1x TAE or 1x TBE.

23. *Dpn*I restriction endonuclease.

24. Chemo-competent MH1 strain (see section 2.1).

25. SOB medium, LB medium and LB-agar plates as in section 2.1.

26. Incubation shaker.

27. Restriction endonuclease recognizing the site introduced into the MUT-F/MUT-R primers (*see* **Note 4**).

28. Set of primers for sequencing of the insert cloned into pET28-6xHis-SUMOTag (unique for a given insert).

2.3 Heterologous expression of the exonuclease in bacteria

1. Chemo-competent *E. coli* BL21-CodonPlus-RIL strain (Stratagene) (*see* **Note 9**).

2. LB-agar plates as in section 2.1
3. Standard, sterile LB medium supplemented with 50 µg/mL of kanamycin and 34 µg/mL of chloramphenicol.
4. 100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) prepared right before use by dissolving 0.24 g of IPTG in 10 mL of sterile water.
5. Incubation shaker.
6. Refrigerated centrifuge.

2.4 Purification of 6xHis-SUMO-tagged exonuclease

1. Lysis buffer: 20 mM Tris-HCl pH 8, 200 mM NaCl, 10 mM imidazole, 10 mM 2-mercaptoethanol, 1 mM PMSF, 2 µM pepstatin A, 0.6 µM leupeptin, 2 mM benzamidine, 2 µg/mL chymostatin.
2. Sonicator (e.g., Bioruptor® XL sonicator from Diagenode) with accessories (*see* **Note 10**).
3. Ultracentrifuge.
4. 1 mL Ni-NTA Superflow Cartridge column (Qiagen).
5. Automated, FPLC-type chromatography system.
6. Standard 96-well plates.
7. Buffer A: 20 mM Tris-HCl pH 8, 200 mM NaCl, 10 mM imidazole, 10 mM 2-mercaptoethanol.
8. Buffer B: 20 mM Tris-HCl pH 8, 1 M NaCl, 10 mM imidazole, 10 mM 2-mercaptoethanol.
9. Buffer C: 20 mM Tris-HCl pH 8, 200 mM NaCl, 600 mM imidazole, 10 mM 2-mercaptoethanol.
10. Buffer D: 20 mM Tris-HCl pH 8, 1 M NaCl, 600 mM imidazole, 10 mM 2-mercaptoethanol.
11. SUMO protease.

12. Dialysis tubing with a proper molecular weight cut-off.
13. Precast 10-12% SDS-PAGE gels with adequate electrophoresis system and power supply.
14. Staining solution for SDS-PAGE gels: 0.025% (w/v) Coomassie Brilliant Blue R-250, 7.5% (v/v) acetic acid, 40% (v/v) methanol.
15. De-staining solution for SDS-PAGE gels: 4% (v/v) acetic acid, 10% (v/v)methanol or ethanol.
16. Superdex^{TM} 75 100/300 GL or Superdex^{TM} 200 10/300 GL size exclusion column (GE Healthcare).
17. GF buffer: 10 mM Tris-HCl pH 8, 150 mM NaCl.
18. RNase-free glycerol.
19. Liquid nitrogen.

2.5 Preliminary analysis of exoribonucleolytic activity

1. Linearized, gel-purified plasmid DNA template (*see* **Note 11**).
2. 10x RNAPol Reaction Buffer (New England Biolabs).
3. 100 mM DTT.
4. NTP Mix: 2.5 mM ATP, 2.5 mM GTP, 2.5 mM CTP, and 0.25 mM UTP in RNase-free water.
5. RiboLock^{TM} RNase Inhibitor (Thermo Scientific).
6. 3000 Ci/mmol [α-^{32}P]-UTP.
7. T7 (or SP6) RNA polymerase (*see* **Note 11**).
8. 1/1 (v/v) Phenol/chloroform mix.
9. Sephacryl^{TM} S-200 solution (GE Healthcare).
10. Spin modules with catch tubes (MP Biomedicals).
11. TE buffer: 10 mM Tris-HCl pH 8, 1 mM EDTA.

12. Hand-held radioactivity detector.
13. RNA dilution buffer: 10 mM Tris-HCl pH 8, 20 mM KCl.
14. 0.5 M EDTA, pH 8.0.
15. Exonuclease reaction buffer: 10 mM Tris-HCl pH 8, 90 mM NaCl, 170 µM $MgCl_2$, 2 mM 2-mercaptoethanol (*see* **Note 12**).
16. Liquid nitrogen (in a styrofoam box or in a thermos).
17. TLC PEI Cellulose plates 20 x 20 cm. Cut the plate into two parts (20 x 10 cm) and, using a thin line marker, draw a line parallel to the longer (20 cm) edge of the plate (at a distance of approximately 1.5 cm from the edge), on its glossy side, not covered with PEI Cellulose matrix. Using the marker, draw reasonably large dots along the line, separated by at least 0.75 cm distance from each other. The line with dots should be visible from the other side (through the plate).
18. TLC developing solution: 1 M formic acid, 0.5 M LiCl.
19. A tray to be filled with TLC developing solution or a chromatography chamber.
20. Phosphorimager (e.g., FLA-3000 imager from Fuji) with phosphorimaging plates and cassettes.

2.6 Purification of the synthetic RNA oligonucleotide substrate

1. Materials 2-15 from section 2.2.
2. RNA elution buffer: 100 mM Tris-HCl pH 7.5, 150 mM NaCl, 12.5 mM EDTA, 1% SDS.
3. 1/1 (v/v) Phenol/chloroform mix.
4. Rotating wheel.
5. 2-propanol
6. Microvolume spectrophotometer.

2.7 5'-end labeling of synthetic RNA oligonucleotides

1. 150 pmol/µL, PAGE-purifed RNA oligonucleotide in sterile RNase-free water.
2. T4 polynucleotide kinase (PNK) enzyme with 10x PNK reaction buffer.
3. 3000 Ci/mmol [γ-^{32}P]-ATP.
4. Sterile RNase-free water.

2.8 3'-end labeling of synthetic RNA oligonucleotides

1. 150 pmol/µL, PAGE-purifed RNA oligonucleotide in sterile RNase-free water.
2. 3.75 mM Cytidine-3'-monophosphate (3'-CMP) in sterile RNase-free water.
3. T4 polynucleotide kinase (PNK) enzyme with 10x PNK reaction buffer.
4. 3000 Ci/mmol [γ-^{32}P]-ATP.
5. 1/1 (v/v) Phenol/chloroform mix.
6. 100% DMSO.
7. 1 mM ATP.
8. 100 mM DTT.
9. RiboLock™ RNase Inhibitor (Thermo Scientific; 40 U/µL) or equivalent.
10. T4 RNA ligase I with 10x ligase reaction buffer.

2.9 Preparation of labeled circular RNA oligonucleotide

1. 150 pmol/µL, PAGE-purifed RNA oligonucleotide in sterile RNase-free water.
2. Sterile RNase-free water.
3. 500 mM Tris-HCl pH 8.0.
4. 50 mM $MgCl_2$.

5. 100 mM DTT.
6. 100 µM ATP.
7. 3000 Ci/mmol [γ-^{32}P]-ATP.
8. 40 U/µL RiboLock™ RNase Inhibitor (Thermo Scientific).
9. T4 PNK enzyme and 10x reaction buffer.
10. T4 RNA ligase I and 10x reaction buffer.

2.10 Preparation of double-stranded RNA substrate

1. 150 pmol/µL, PAGE-purifed RNA oligonucleotide 1 in sterile, RNase-free water (*see* **Note 13**).
2. 150 pmol/µL, PAGE-purifed complementary RNA oligonucleotide 2 in sterile, RNase-free water (*see* **Note 14**).
3. 5'-labeled RNA oligonucleotide 1, prepared as described in section 3.7.1.
4. RNA dilution buffer: 10 mM Tris-HCl pH 8, 20 mM KCl.
5. 15% native polyacrylamide stock – acrylamide:bisacrylamide (19:1) in 1x TBE buffer (*see* **Note 5**). Filter with a 0.2 filter unit and store at 20°C in a glass bottle wrapped with aluminum foil to protect from light.
6. 15% native polyacrylamide gel (10 cm x 8 cm) prepared from 15% native polyacrylamide stock with 1 mm thick spacers and 0.3 cm-teeth comb as described in section 2.2.
7. Vertical gel electrophoresis system.
8. Native gel loading dye: 10 mM Tris-HCl pH 8.0, 60% (v/v) glycerol, 60 mM EDTA, 0.03% (w/v) bromophenol blue, 0.03% (w/v) xylene cyanol.
9. Phosphorimager with phosphorimaging plates and cassettes.

2.11 Purification of labeled RNA oligonucleotides

1. 1/1 (v/v) Phenol/chloroform mix.
2. Materials 2-9 and 11-15 from section 2.2.
3. Autoradiography film (such as Kodak BioMax or equivalent).
4. RNA elution buffer (see section 2.6).
5. 2-propanol.
6. 20 mg/mL RNase-free glycogen.
7. Hand-held radioactivity detector.
8. 10% denaturing polyacrylamide gel (10 cm x 8 cm) prepared with 1 mm thick spacers and 0.3 cm-teeth comb as described in section 2.2.
9. Vertical gel electrophoresis system.
10. Phosphorimager with phosphorimaging plates and cassettes.

2.12 Gel-based exoribonuclease activity assay with 5'-labeled RNA substrate

1. 20% denaturing polyacrylamide gel prepared with 1 mm thick spacers and 0.3 cm-teeth comb as described in section 2.2 (*see* **Note 15**).
2. Vertical gel electrophoresis system.
3. F-dye (see section 2.2).
4. Exonuclease reaction buffer: 10 mM Tris-HCl pH 8.0, 90 mM NaCl, 170 µM $MgCl_2$, 2 mM 2-mercaptoethanol (*see* **Note 12**).
5. Liquid nitrogen (in a styrofoam box or in a thermos).
6. 1x TBE buffer.
7. Saran.
8. Phosphorimager with phosphorimaging plates and cassettes.

2.13. XRN1 5'-3' exoribonuclease activity assay with RNA substrates having different 5'-end phosphorylation status and 3'-end fluorescent labels

1. 200 µM stocks of synthetic RNA oligonucleotide 5'ACUCACUCACUCACCAAAAAAAAAAAAACC (*see* **Note 16**) containing, at the 5'-terminus, a monophosphate (5'-P), triphosphate (5'-PPP), hydroxyl group (5'-OH) or methylated cap (5'-m^7Gppp) and labeled with fluorescein amidite (FAM) at the 3'-end. Each oligonucleotide batch is purified by RNase-free dual HPLC and PAGE (*see* **Note 17**).
2. Sterile RNase-free water.
3. 1 U/µL XRN1 (New England Biolabs).
4. 20% denaturing polyacrylamide gel prepared with 1 mm thick spacers and 0.25 cm-teeth comb as described in section 2.2 (*see* **Note 18**).
5. Vertical gel electrophoresis system.
6. F-dye (see section 2.2).
7. 10x NEBuffer 3 (New England Biolabs).
8. 40 U/µL RiboLock™ RNase Inhibitor (Thermo Scientific).
9. Liquid nitrogen (in a styrofoam box or in a thermos).
10. 1x TBE buffer.
11. High-voltage DC power supply.
12. Saran.
13. Phosphorimager with fluorescence detection capability (e.g., Typhoon™ FLA-9000 biomolecular imager from GE Healthcare).

3. Methods

3.1. Preparation of the expression vector coding for the exonuclease of interest with an N-terminal 6xHis-SUMOTag

There are various ways to prepare a plasmid construct bearing the coding sequence of an exonuclease in a way allowing its efficient heterologous expression in bacteria and its purification in a soluble form, which is a prerequisite for successful biochemical *in vitro* assays. In our laboratory, we routinely use a derivative of pET28a plasmid with SUMOTag (pET28M-6xHis-SUMOTag). We utilize the sequence and ligation independent cloning (SLIC) procedure to introduce the open reading frame corresponding to the protein of interest in frame with the N-terminal 6xHis-SUMOTag. In our hands SLIC works much more efficiently than standard cloning strategies. Besides, the linearized acceptor plasmid may be employed for various inserts without the need of fresh preparation before each cloning attempt. Furthermore, we have found that the N-terminal 6xHis-SUMOTag significantly increases the solubility of numerous proteins, including exonucleases, such as DIS3 protein homologs from different species. Below we present our protocol for SLIC.

3.1.1 Preparation of the insert

1. Amplify the sequence coding for the exonuclease of interest using the SLIC-F and SLIC-R pair of oligonucleotides and a DNA polymerase with a high fidelity rate such as Phusion to avoid mutations. Mix 10 µL of 5x Phusion HF buffer, 1.5 µL of DMSO, 1 µL of 10 mM dNTP mix, 1 µL of the SLIC-F primer (5 pmol/µL), 1 µL of the SLIC-R primer (5 pmol/µL), 100 ng of the plasmid containing the DNA fragment encompassing the open reading frame coding for the exonuclease of interest (*see* **Note 1**), 0.5 µL of the Phusion High-Fidelity DNA Polymerase (2 U/µL), and sterile water to 50 µL. To increase the amount of insert, prepare several (4-6) identical reaction mixtures from a master mix.

2. Place the mixtures in a thermocycler and run the following PCR program (*see* **Note 19**): 98°C for 3 min (initial denaturation) followed by 35 cycles of denaturation at 98°C for 10 s, hybridization at 55°C for 30 s, and elongation at 72°C for 30 s per kilobase of insert (*see* **Note 20**) and by a final elongation step at 72°C for 10 min.
3. Combine mixtures in one 1.5 mL microtube and use 5 µL to check size of the PCR products on a standard 1% agarose gel followed by ethidium bromide staining (*see* **Note 21**).
4. Extract the remainder of the PCR mixture with an equal volume of phenol/chloroform/isoamyl alcohol. Centrifuge at 21000 x g for 5 min at room temperature.
5. Transfer the upper aqueous phase to a new 1.5 mL microtube and extract with one volume of chloroform; Centrifuge at 21000 x g for 5 min at room temperature.
6. Transfer the upper aqueous phase to a new 1.5 mL microtube, add 0.1 volume of 3 M sodium acetate (pH 5.2) and precipitate DNA with 2.5 volumes of 96% ethanol. Centrifuge at 21000 x g for 5 min at 4°C. Discard supernatant.
7. Wash the pellet with 1 mL of 75% ethanol and centrifuge at 21000 x g for 5 min at 4°C.
8. Remove supernatant and leave the tube opened for 5 min at room temperature to air-dry the pellet (*see* **Note 22**).
9. Suspend the pellet in 40 µL of sterile water.
10. Add 8 µL of the 6x Orange G loading dye to the concentrated PCR products and load 12 µL of the mixture into each of four wells of a 1% agarose gel containing GelGreen™ (*see* **Note 23**).
11. After electrophoresis for 1 h at 7 V/cm, place the gel on a transilluminator and excise the appropriate bands with a sterile scalpel. Use a commercial, spin column-based gel

extraction kit to recover DNA, following manufacturer's instructions. About 35 µL of DNA sample should be recovered at this stage.

12. Use ~3 µL of sample to check the purity and estimate the concentration of purified insert by standard 1% agarose gel electrophoresis using known quantities of molecular weight marker as reference (*see* **Note 24**).

3.1.2 Preparation of the vector

1. Digest approximately 4 µg of pET28M-6xHis-SUMOTag vector with the *Bam*HI and *Xho*I enzymes in a total volume of 20 µL, following the recommendations of the manufacturer of the enzymes.
2. Adjust the volume to 200 µL with sterile water and extract with one volume of phenol/chloroform/isoamyl alcohol. Centrifuge at 21000 x g for 5 min at room temperature.
3. Phenol-extract and ethanol-precipitate the mixture as described in section 3.1.1, steps 4–8. Resuspend the DNA pellet in 20 µL of sterile water.
4. Add 4 µL of the 6x Orange G loading dye to the concentrated linearized vector and load 6 µL of the mixture into each of four wells of a preparative 1% agarose gel containing GelGreen™ (*see* **Note 23**).
5. After electrophoresis for 1 h at 7 V/cm, place the gel on a transilluminator and excise the appropriate bands with a sterile scalpel. Use a commercial, spin column-based gel extraction kit to recover DNA, following manufacturer's instructions. Eventually, elute DNA from the column using 20 µL of sterile water pre-warmed to 70°C.

6. Use ~3 µL of sample to check the purity and estimate the concentration of purified linearized vector by standard 1% agarose gel electrophoresis using known quantities of molecular weight marker as reference (*see* **Note 24**).

3.1.3 SLIC reaction

1. Mix 17.5 µL of purified insert (section 3.1.1, step 11), 2 µL of NEBuffer 2, 0.3 µL of BSA, and 0.2 µL of T4 DNA polymerase and incubate for 30 minutes at 22°C. Then, add 2 µL of any 10 mM dNTP (for instance, dGTP) and place on ice.
2. In another microtube, mix 100 ng of linearized vector (section 3.1.2, step 5), 1.5 µL of NEBuffer 2, 0.3 µL of BSA, 0.2 µL of T4 DNA polymerase, and sterile water to 15 µL and incubate for 30 minutes at 22°C. Then, add 1.5 µL of any 10 mM dNTP (for instance, dGTP) and place on ice.
3. Combine both insert and vector mixtures and incubate for 7 min at 44°C in a thermoblock. Then, switch off heating and wait until the device cools down to room temperature (*see* **Note 25**). Place the tube on ice (*see* **Note 26**).

3.1.4 Transformation of bacteria with SLIC products and selection of transformants

1. Transform an aliquot of chemo-competent MH1 cells (*see* **Note 27**) with 20 µL of the SLIC products. Place the tube containing the mixture of bacteria and DNA on ice for 30 minutes, incubate it for exactly 90 seconds at 42°C in a thermoblock, and immediately add 1 mL of SOB medium. Incubate with shaking at 37°C for 60 minutes to express kanamycin resistance. Streak on LB-kanamycin plates and incubate overnight at 37°C.
2. Pick several (6-10) colonies with sterile toothpicks and use each of them to inoculate 5 mL of LB medium supplemented with kanamycin. Incubate overnight at 37°C with shaking.

3. Use 3 mL of each bacterial culture to isolate plasmids using a commercial mini-preparation kit or other method of choice.
4. Digest the purified plasmids with *Bam*HI and *Xho*I enzymes following manufacturer's instructions. Analyze the digestion products by standard 1% agarose gel electrophoresis to validate the presence of the properly-sized insert.
5. Verify insert sequence in selected plasmid(s) using a commercial sequencing service or core facility. Make sure that unwanted mutations are absent.

3.2 Construction of the expression vector encoding the exonuclease with an inactivating mutation in the catalytic site

Bacterial cells used for heterologous expression of eukaryotic exonucleases also contain ribonucleases, which may co-purify with the protein of interest and interfere with correct interpretation of the subsequent biochemical assays. Thus, it is absolutely necessary to have a proper negative control. Bacteria transformed with an empty expression vector may be used at initial stage of analysis. However, it is strongly advisable to prepare a vector expressing an active site mutant of the exonuclease under study, provided that the mutation abolishes the exoribonucleolytic activity of the protein. To this end, one should use all structural and mechanistic information available to determine which residue(s) is the best target(s) for site-directed mutagenesis.

3.2.1 Purification of primers for site-directed mutagenesis

To achieve a high efficiency of mutagenesis it is absolutely crucial to use only full-length primers during amplification step. From our experience, it is evident that the presence of shorter by-products of oligonucleotide synthesis increases the risk of introducing unwanted

mutations, especially short deletions, around the site of mutagenesis, which can make selection of the correct clone extremely time-consuming. Therefore, we recommend ordering oligonucleotide primers purified by HPLC and/or to purify them by denaturing PAGE, as outlined below.

1. Design and order 40 nmoles of MUT-F and MUT-R primers (*see* **Note 4**). Resuspend each oligonucleotide in 200 µL of sterile water.
2. Transfer 50 µL of each of the oligonucleotide solutions into separate 1.5 mL microtubes and add 25 µL of F-dye. Heat-denature the mixtures for 5 min at 90^{o}C and then place on ice. Spin down briefly.
3. Prepare a 10% denaturing polyacrylamide gel (20 cm x 20 cm) and install it in a vertical electrophoresis system with tanks filled with 1x TBE. Rinse the gel wells thoroughly with 1x TBE to remove diffusing urea right before loading the samples.
4. Load the content of each tube into a gel well and then run the gel at a constant power of 15 W until the bromophenol blue (lower dye) has reached approximately 2 cm from the bottom edge of the gel (*see* **Note 28**).
5. Gently dismantle the glass plates; the gel should remain attached to only one of the plates (*see* **Note 29**).
6. Cover the gel with the saran wrap.
7. Use a 254 nm hand-held UV lamp in a darkroom to visualize the position of the full-length oligonucleotide band(s) by UV-shadowing (*see* **Note 30**).
8. Mark the location(s) of the band(s) on the saran wrap or on the reverse side of the glass plate using a thin line marker.

9. Cut out the corresponding gel band(s) with a sterile scalpel blade (one for each gel band(s)).
10. Put each gel band into a 1.5 mL microtube and centrifuge at 21000 x g in a microcentrifuge at room temperature.
11. Prepare pestle(s) for grinding the gel band(s) by gently melting the end of 1000 µL micropipette tip(s) over the flame of laboratory burner or lighter. Wait until the plastic cools down and becomes opaque.
12. Snap-freeze the tube(s) with the gel band(s) in liquid nitrogen, then remove with forceps and immediately open lid(s). Incubate at room temperature for approximately 90 seconds. Using the pestle(s), grind the gel band(s) until homogeneity.
13. Add 1 mL of DNA extraction buffer to each tube. Incubate overnight at 37^{o}C with shaking (*see* **Note 31**).
14. Centrifuge at 21000 x g for 5 min at room temperature and transfer supernatant(s) into new microtube(s).
15. Repeat step 14 to get rid of as much of acrylamide as possible.
16. Divide supernatant(s) into two microtubes (approximately 450 µL into each tube) and precipitate DNA by adding 1 mL of 2-propanol into each tube and incubating overnight at -20^{o}C (*see* **Note 32**).
17. Centrifuge at 21000 x g for 15 min at 4^{o}C. Wash the pellets with 70% ethanol and centrifuge again. Remove supernatant(s).
18. Open the tubes and allow pellets to dry for 5 min at room temperature (*see* **Note 22**).
19. Resuspend each pellet in 25 µL of sterile water and combine the contents of both precipitation tubes (step 16) so that the total volume is 50 µL.
20. Measure DNA concentration using a microvolume spectrophotometer (*see* **Note 33**).

21. Prepare a 250 ng/µL working dilution for each oligonucleotide.

3.2.2 Site-directed mutagenesis

1. Mix 100 ng of expression plasmid (containing the wild-type exonuclease sequence; see section 3.1.4), 10 µL of 5x Phusion HF buffer, 1.5 µL of DMSO, 1 µL of 10 mM dNTP mix, 1 µL of the purified MUT-F primer (250 ng/µL), 1 µL of the purified MUT-R primer (250 ng/µL), 0.5 µL of the Phusion High-Fidelity DNA polymerase (2 U/µL) and sterile water to 50 µL.
2. Place the mixtures in a thermocycler and run the following PCR program (*see* **Note 34**): 98^{o}C for 3 min (initial denaturation) followed by 35 cycles of denaturation at 98^{o}C for 10 s, hybridization at 55^{o}C for 30 s, and elongation at 72^{o}C for 60 s per kilobase of insert and by a final elongation step at 72°C for 10 min.
3. Use 5 µL of the mixture for analysis on a standard 1% agarose gel to verify that the amplification worked (*see* **Note 35**).
4. Transfer 10 µL of the mixture into a new 1.5 mL microtube and digest the template with 20 units of *Dpn*I enzyme in a final volume of 20 µL, following manufacturer's instructions (*see* **Note 36**).
5. Transform an aliquot of chemocompetent MH1 cells with 10 µL of the *Dpn*I-treated sample, as described in section 3.1.4, step 1. Isolate plasmid clones as described in section 3.1.4, steps 2-3.
6. Digest the purified plasmids using the restriction enzyme recognizing the sequence introduced into the MUT-F/MUT-R primers during their design (*see* **Note 4**). Using gel agarose electrophoresis, compare the restriction pattern with that of the wild-type

template digested in the same way; make sure that the patterns differ and that the bands with the expected sizes are observed (*see* **Note 37**).

7. Verify insert sequence in selected plasmid(s) using a commercial sequencing service or core facility. Make sure that no mutations other than the one introduced during site-directed mutagenesis occurred.

3.3 Heterologous expression of the exonuclease of interest in bacteria

1. Transform an aliquot of chemocompetent *E. coli* BL21-CodonPlus-RIL cells (*see* **Note 9**) with 100 ng of plasmid DNA as described in section 3.1.4, step 1. Use plasmids encoding both wild-type (section 3.1.4) and mutant (section 3.2.2) exonuclease in parallel. Streak on LB-kanamycin plates and incubate overnight at 37^{o}C.
2. In each case, scrap several colonies with an inoculation loop and place them into 300 mL Erlenmeyer flasks containing 100 mL of LB medium supplemented with kanamycin and chloramphenicol. Incubate overnight at 37^{o}C with shaking.
3. Dilute 30 mL of the pre-culture from step 2 into 1000 mL of LB medium supplemented with kanamycin and chloramphenicol (*see* **Note 38**). Incubate until OD_{600} reaches 0.45-0.5 and then move the flasks to 18^{o}C (*see* **Note 39**); continue shaking the cultures for 30 minutes to cool them down (*see* **Note 40**).
4. Induce protein expression by adding 1/100th volume of IPTG, so that the final concentration is 1 mM and incubate overnight at 18^{o}C with shaking (*see* **Note 39**).
5. Harvest the cells by centrifugation at 4500 x g for 5 min at 4^{o}C. At this stage the pellet may be frozen at -20^{o}C for later use.

3.4 Purification of the N-terminally 6xHis-SUMO-tagged exonuclease

This section is dedicated to the protocol that should be employed for purification of any given 6xHis-SUMO-tagged protein, unless the protein is entirely insoluble following heterologous expression. It is based on two rounds of affinity chromatography on a Ni-NTA resin, separated by the cleavage of 6xHis-SUMOTag with SUMO protease (**Fig. 2a**). At the end of the first round, the protein of interest equipped with 6xHis-SUMOTag is released from the resin during the elution step. In the second round the protein is devoid of 6xHis-SUMOTag (and thus no longer binds to the column) and predominates in the flow-through fraction whereas 6xHis-SUMOTag and SUMO protease (also bearing a 6xHis epitope) remain associated with the Ni-NTA resin (**Fig. 2a**). This experimental setup eliminates contaminating proteins, which may potentially co-purify on the nickel resin with the protein of interest. Usually, another purification method, such as gel filtration, is then sufficient to purify the exonuclease under study to near homogeneity. *[Note to publisher: Figure 2 near here]*

1. Resuspend cell paste (see section 3.3, step 5) in 10 mL of the lysis buffer, by pipetting and/or gently vortexing. Adjust volume to 15 mL using lysis buffer and then divide mixture into three 50 mL Falcon tubes. Place the tubes on ice.
2. Sonicate samples on ice using high power setting and applying 30 seconds on/off cycles for 45 minutes (*see* **Note 10**). Conditions may vary with the type of sonicator used.
3. Centrifuge cell debris at 120000 x g for 45 min at 4^{o}C in an ultracentrifuge.
4. Collect supernatant (approximately 15 mL), corresponding to a total native protein extract. Save 20 µL of the extract for SDS-PAGE analysis (**Fig. 2b**, lane EX).
5. Install the 1 mL Ni-NTA Superflow Cartridge column on an automated chromatography system and equilibrate it with buffer A at a flow rate of 2 mL/min.

6. Load the protein extract on the column and collect three 1.5 mL fractions of the flow-through into the wells of 96-well 2 mL Masterblock plate to assess the efficiency of binding of the tagged protein to the nickel resin. Combine fractions and save them for SDS-PAGE analysis (**Fig. 2b**, lane FT1); wash with 20 mL of buffer A (*see* **Note 41**).
7. Wash the column with 10 mL of buffer B and then with 10 mL of buffer A. Elute the protein with 10 mL of buffer C (*see* **Note 41**). Collect ten 0.5 mL fractions of the eluate into the wells of 96-well 2 mL Masterblock plate; save 20 µL of eluate for SDS-PAGE analysis (**Fig. 2b**, lane EL1).
8. Wash the column with 5 mL of buffer C (*see* **Note 41**).
9. Combine fractions 3-8 of the eluate collected in step 7 (3 mL in total) into a 15 mL Falcon tube and add SUMO protease (10 µg/mL) (*see* **Note 42**).
10. Transfer the mixture from step 9 into the appropriate dialysis tubing and dialyze it against 2 liters of buffer A overnight at 4^{o}C in order to cleave off the 6xHis-SUMOTag from the exonuclease of interest and at the same time to reduce imidazole concentration to 10 mM. Save a 20 µL aliquot for SDS-PAGE analysis (**Fig. 2b**, lane DIAL).
11. Equilibrate the 1 mL Ni-NTA Superflow Cartridge column (*see* **Note 43**) with buffer A and load the mixture onto the column using buffer A as the eluent. Collect ten 0.5 mL fractions of the flow-through and combine fractions 2-10 together (4.5 mL in total) – these constitute the most important sample, as they contain the exonuclease of interest separated from the 6xHis-SUMOTag and SUMO protease (**Fig. 2b**, lane FT2).
12. Elute the bound proteins (mainly 6xHis-SUMOTag and SUMO protease) with 10 mL of buffer D (*see* **Note 41**); collect eight 0.5 mL fractions of the eluate into the wells of 96-well 2 mL Masterblock plate. Combine fractions 3-6 for SDS-PAGE analysis (**Fig. 2b**, lane EL2).

13. Analyze the various samples collected during purification by SDS-PAGE. A representative gel obtained during purification of *A. thaliana* DIS3 protein is shown in **Fig. 2b**.
14. To further purify the exonuclease, load 1 mL of sample FT2 (step 11) on a Superdex^{TM} 75 100/300 GL or Superdex^{TM} 200 10/300 GL size exclusion column (depending on the size of the investigated protein) that had been pre-equilibrated with GF buffer. Collect 400 µL fractions into the wells of 96-well 2 mL Masterblock plate while monitoring absorbance at 215 nm. Transfer fractions corresponding to the most intense peak of the chromatogram (**Fig. 2a**) into 1.5 mL microtubes.
15. Following analysis by SDS-PAGE, combine fractions that are both the richest and purest in exonuclease (*see* **Note 44**). Add 0.33 volume of RNase-free glycerol. Mix by gentle vortexing and divide the solution into 100 µL aliquots.
16. Freeze aliquots in liquid nitrogen and store at -80^{o}C (see **Note 45**).

3.5 Preliminary analysis of the exoribonucleolytic activity using uniformly-labeled RNA substrate and thin-layer chromatography

The most accurate way to assess biochemical features of the purified exonuclease (e.g., directionality, processivity, etc.) is by using the methods described in sections 3.7 to 3.9. However, these methods are effort/time-consuming and it is often useful to perform a simpler analysis beforehand, to tentatively demonstrate that the wild-type protein is active whereas its mutated counterpart is not (or has an activity that is significantly reduced). To this end, we use an internally-labeled RNA substrate obtained by *in vitro* transcription in the presence of [α-^{32}P]-UTP. Irrespective of enzyme directionality, exonuclease-mediated degradation of such RNA will release [α-^{32}P]-UMP which is easily detected by TLC. A detailed protocol is provided below.

3.5.1 Preparation of the uniformly-labeled RNA substrate

1. In a laboratory suitable for handling radiomaterials, mix 3 µL of 10x RNAPol Reaction Buffer, 3 µL of 100 mM DTT, 3 µL of NTP Mix, 0.5 µg of gel-purified, linearized plasmid (*see* **Note 11**), 1.5 µL of RiboLock™ RNase Inhibitor, 3 µL of [α-^{32}P]-UTP, 1.5 µL of 50 U/µL T7 (or 20 U/µL SP6) RNA polymerase, and sterile RNase-free water to 30 µL. Incubate at 37°C for 90 min and then add 0.75 µL of T7 (or SP6) RNA polymerase. Incubate at 37°C for 30 min.
2. Inactivate the enzyme by incubating the mixture at 90°C for 5 min. Place the tube on ice for 30 seconds and then spin down briefly.
3. Add 30 µL of phenol/chloroform and vortex for 30 seconds. Centrifuge at 21000 x g for 5 min at room temperature and collect the upper aqueous phase.
4. Pack a Spin Module with Sephacryl S-200 resin equilibrated in TE buffer. Load sample in the Spin Module and centrifuge at 1000 x g for 3 min.
5. Estimate the radioactivity in the Spin Module eluate with a hand-held radioactivity detector.
6. Use RNA dilution buffer to prepare an aliquot of labeled RNA representing approximately 1500-2000 cps of radioactivity in a final volume of 100 µL.

3.5.2 Preliminary exoribonuclease activity assay and TLC analysis

1. Prepare 1.5 mL microtubes, each containing 0.5 µL of 0.5 M EDTA (*see* **Note 46**).
2. In a 1.5 mL microtube, combine 10 µL of the appropriate reaction buffer (*see* **Note 12**) and 5 µL of the protein solution in GF buffer, purified as described in section 3.4 (in our case, approximately 2 pmol of protein) (see **Note 47**). Place on ice.

3. Add 5 µL of the labeled RNA oligonucleotide solution (section 3.5.1, step 6), mix by pipetting, and transfer 5 µL of the mixture into the first tube containing EDTA. Freeze this t0 sample in liquid nitrogen (*see* **Note 48**). Incubate the remainder of the reaction mixture at 37°C (*see* **Note 49**).
4. At desired reaction times, remove 5 µL reaction aliquots and mix them with EDTA in the preset tubes (step 1). Freeze each quenched aliquot in liquid nitrogen (*see* **Note 48**).
5. After collecting the last sample, take the tubes one by one out of the liquid nitrogen. Open tubes to avoid popping due to the rapid change of temperature and leave them open at room temperature for 2 min.
6. Spot 1 µL aliquots (equal to approximately 3-5 cps of radioactivity) from each of the collected samples onto the PEI Cellulose TLC plate (samples taken at consecutive time points for each reaction condition should be analyzed side by side, starting from t0; **Fig. 3**). The leftmost and rightmost spots should not be closer than 1 cm to the edges of the TLC plate (**Fig. 3**).
7. Wait until all spots are completely dry (*see* **Note 50**).
8. Develop the plate by evenly placing the bottom edge of the TLC plate in a tray containing developing solution (*see* **Note 51**). Maintain the plate vertical during development and allow the solvent front to migrate up to ~2 cm from the top of the TLC plate.
9. Allow the developing solution to evaporate completely from the plate (*see* **Note 52**). Cover plate with saran-wrap and expose it to a phosphorimaging screen overnight.
10. Scan the screen with a phosphorimager. A representative example of TLC image obtained upon analysis of different variants of the human DIS3 protein is shown in **Fig. 3** (*see* **Note 53**). *[Note to Publisher: Figure 3 near here].*

3.6 Purification of the synthetic RNA oligonucleotide substrate

Analysis of exonuclease activity requires relatively short (20-100 nt) RNA oligonucleotides labeled in different ways that are used as substrates in the assays. Prior to labeling, it is best to PAGE-purify synthetic RNA oligonucleotides as described below.

1. Order 200 nmoles of the RNA oligonucleotide to be used as substrate in the enzymatic assays (*see* **Note 54**) from a commercial source. Resuspend in 100 µL of sterile RNase-free water.
2. Mix 25 µL of the oligonucleotide solution with 25 µL of F-dye (store the remainder of RNA oligonucleotide solution at -80^{o}C in 25 µL aliquots).
3. Incubate mixture at 90^{o}C for 5 min, place on ice, and then spin down briefly.
4. Proceed as described in section 3.2.1, steps 3 to 12.
5. Add 650 µL of RNA elution buffer and 300 µL of phenol/chloroform to the homogenized gel piece. Incubate overnight at room temperature on a rotating wheel.
6. Centrifuge the acrylamide at 21000 x g for 3 min at room temperature and carefully transfer the upper aqueous phase into a new microtube.
7. Precipitate purified RNA oligonucleotide by adding 1 mL of 2-propanol and incubating overnight at -20^{o}C.
8. Centrifuge at 21000 x g for 15 minutes at 4^{o}C and remove supernatant. Wash the pellet with 80% ethanol and spin down again.
9. Open the tube and allow pellet to dry for 5 minutes at room temperature (*see* **Note 22**).
10. Dissolve the pellet in 25 µL of sterile water and determine RNA concentration by measuring absorbance at 260 nm with a microvolume spectrophotometer.
11. Prepare a working dilution of the RNA oligonucleotide (150 pmol/µL) with RNase-free water.

3.7 Preparation of synthetic RNA oligonucleotide substrates labeled in different ways

In this section, we describe the preparation of the various RNA substrates required for detailed analysis of ribonuclease features. Only the combination of assays performed with substrates labeled in different manners will provide the researcher with unambiguous information about the directionality of the exonuclease, the presence (or lack) of accompanying endonuclease activity, processivity, length/distribution of the degradation products, and specificity towards single-stranded versus structured substrates.

3.7.1 Labeling of the synthetic RNA oligonucleotide substrate at the 5'-end and purification of the labeled substrate

1. In a ^{32}P-dedicated laboratory, mix 5 µL of sterile RNase-free water, 1 µL of 10x T4 PNK buffer, 1 µL of T4 PNK enzyme, 1 µL of purified RNA oligonucleotide (section 3.6, step 11), and 2 µL of [γ-^{32}P]ATP.
2. Incubate for 60 min at 37°C and then for 5 min at 90°C. Place the tube on ice for 30 seconds and then spin down briefly.
3. Add 20 µl of phenol/chloroform, vortex for 30 seconds, and centrifuge at 21000 x g for 5 min at room temperature.
4. Transfer aqueous upper phase to a new tube and add 20 µL of F-dye. Incubate for 5 minutes at 90°C and then place on ice.
5. Spin down briefly and load the entire mixture into the flushed well of a preparative 10% denaturing polyacrylamide gel (see section 3.2.1). Run the gel at a constant power of 15 W until the bromophenol blue (lower dye) is approximately 5 cm from the bottom edge of the gel.

6. Gently dismantle the glass plates (the gel should remain attached to only one of the plates). Cover the gel with saran wrap and place it behind a shielding Plexiglas screen efficiently stopping beta-radiation emitted by ^{32}P.
7. In a darkroom, expose the gel to an autoradiography film (mark the position of the film on the gel with a thick permanent marker by drawing several horizontal or diagonal lines crossing the border of the film and the gel). After exposure, develop the film (using a dedicated instrument or manual transfers into developing and fixating bathes).
8. Behind a Plexiglas screen, align the gel with the film utilizing the guide marks and excise the slowest migrating (usually most intense) band from the gel using a nuclease-free scalpel blade.
9. Proceed as described in section 3.2.1 steps 10-12 and then section 3.6 steps 5-6.
10. Precipitate purified labeled RNA oligonucleotide by adding 2 µL of RNase-free glycogen (20 mg/mL) and 1 mL of 2-propanol and by incubating overnight at -80°C. Centrifuge at 21000 x g for 15 minutes at 4°C and remove supernatant.
11. Proceed as described in section 3.6 steps 8-9.
12. Dissolve the pellet in 100 µL of sterile water and estimate sample radioactivity with a hand-held detector.
13. Analyze an aliquot of the purified labeled RNA oligonucleotide (equal to approximately 20 cps) on a small (10 x 8 cm) 10% denaturing polyacrylamide gel. After electrophoresis, remove plates and place the gel on a piece of developed autoradiography film (*see* **Note 55**), cover it with saran-wrap and expose it to a phosphorimaging screen overnight. Ensure that no RNA degradation occurred during purification.

3.7.2 Labeling of the synthetic RNA oligonucleotide substrate at the 3'-end

1. To prepare [5'-^{32}P]cytidine 3',5'-bis(phosphate) (pCp) (see **Note 56**), mix 3 µL of 3.75 mM 3'-CMP, 2.5 µL of sterile RNase-free water, 1 µL of 10x T4 PNK buffer, 1 µL of T4 PNK enzyme, and 2.5 µL of [γ-^{32}P]ATP. Incubate overnight at 37°C.
2. Spin down briefly and extract the mixture with 20 µL of phenol/chloroform by vortexing for 30 seconds. Centrifuge at 21000 x g for 5 minutes at room temperature and transfer the aqueous upper phase containing labeled pCp into a new microtube.
3. Mix 3 µL of sterile RNase-free water, 1 µL of purified RNA oligonucleotide (150 pmol/µL; section 3.6.2, step 11), 2 µL of 10x reaction buffer for T4 RNA ligase I, 2 µL of DMSO, 2 µL of 1 mM ATP, 2 µL of 100 mM DTT, 1 µL of RiboLock RNase Inhibitor, 2 µL of T4 RNA ligase I and 5 µL of [5'-^{32}P]pCp from step 2 (see **Note 56**). Incubate for 2 hours at 37°C.
4. Proceed as described in section 3.7.1 steps 4-13 to purify and check the 3'-labeled substrate.

3.7.3 Preparation of the labeled circular RNA oligonucleotide

1. Mix 2 µL of sterile RNase-free water, 2 µL of 500 mM Tris-HCl, pH 8, 4 µL of 50 mM $MgCl_2$, 2 µL of 100 mM DTT, 1 µL of purified RNA oligonucleotide (150 pmol/µL; section 3.6.2, step 11), 3 µL of 100 µM ATP, 3 µL of [γ-^{32}P]ATP, 1 µL of RiboLock RNase Inhibitor, 1 µL of T4 PNK, and 1 µL of T4 RNA ligase I. Incubate for 2 h at 37°C.
2. Proceed as described in section 3.7.1 steps 4-13 to purify and check the labeled, circularized substrate (*see* **Note 57**).

3.7.4 Preparation of the partially double-stranded RNA substrate (*see* **Note 58**)

1. Prepare the annealing reaction by mixing 400 pmol of purified RNA oligonucleotide 1 with 600 pmol of the purified RNA oligonucleotide 2 and an aliquot of ^{32}P-labeled RNA

oligonucleotide 1 (~3000 cps). Adjust to 500 µL with RNA dilution buffer to reach a concentration of 0.8 pmol/µL.

2. Incubate for 7 minutes at 90°C in a thermoblock. Switch off thermoblock and wait until it cools down to room temperature.
3. Mix 5 µL of the annealing reaction with 5 µL of the native gel loading dye.
4. Prepare a control sample containing approximately the same amount of ^{32}P-labeled RNA oligonucleotide 1 (~3000 cps).
5. Load both samples on a small (10 x 8 cm) 15% native polyacrylamide gel and run gel for 30 min at 30 V/cm.
6. Disassemble the gel cassette, place the gel on a piece of developed autoradiography film (*see* **Note 55**), cover it with saran-wrap and expose it to a phosphorimaging screen overnight.
7. Scan screen with a phosphorimager to ensure proper formation of the duplex substrate (*see* **Note 59**).

3.8 Gel-based assay for exoribonuclease activity using 5'-labeled RNA oligonucleotide substrate

In this section we detail exoribonucleolytic degradation analysis based on the separation of the decay products in a denaturing polyacrylamide gel. Although we use the *A. thaliana* DIS3 protein (which degrades its substrates exonucleolytically in the 3'-5' direction) as an example (**Fig. 4a**), the same assay may be employed to study 5'-3' exoribonucleases. In this latter case, however, the final degradation product will be a single ^{32}P-labeled nucleotide which may be better detected by TLC than by PAGE. Here, we only detail the assay with 5'-labeled

substrate (section 3.7.1) but the same protocol/rules are valid for the other substrates (sections 3.7.2, 3.7.3, and 3.7.4).

1. Prepare a 20% denaturing polyacrylamide gel using 1 mm-thick spacers and comb (*see* **Note 15**).
2. Prepare a substrate master mix containing 800 pmol of unlabeled RNA oligonucleotide (section 3.6) and ~1500 cps of its 5'-labeled counterpart (section 3.7.1). Adjust to 100 µL with RNA dilution buffer to obtain a RNA concentration of 8 pmol/µL (*see* **Note 60**).
3. Prepare 1.5 mL microtubes, each containing 5 µL of F-dye (*see* **Note 46**).
4. In a 1.5 mL microtube, mix 10 µL of the appropriate reaction buffer (*see* **Note 12**) with 5 µL of thawed solution of purified protein (section 3.4, step 16; e.g., ~ 2 pmol of DIS3 protein) (see **Note 47**). Place on ice.
5. Add 5 µL (40 pmol) of substrate master mix (step 2), mix by pipetting, and transfer 5 µL into the first tube containing F-dye (t0 sample) (*see* **Note 48**). Freeze this sample in liquid nitrogen. Incubate the remainder of the reaction mixture at 37^{o}C (*see* **Note 49**).
6. At desired reaction times, remove 5 µL reaction aliquots and mix them with F-dye in the preset tubes (step 3). Freeze each quenched aliquot in liquid nitrogen (*see* **Note 48**)
7. After collecting the last sample, take the tubes one by one out of the liquid nitrogen. Open tubes to avoid popping due to the rapid change of temperature and leave them open at room temperature for 2 min.
8. To denature RNA, incubate tubes at 90^{o}C for 5 min, place on ice, and then spin down briefly.

9. Analyze one-half (5 µL) of each sample by denaturing 20% PAGE (32 x 26 cm gel) (*see* **Note 15**). Perform electrophoresis at 30 W until bromophenol blue (lower dye) reaches ~ 5 cm from the bottom edge of the gel.
10. Disassemble the gel cassette, place the gel on a piece of developed autoradiography film (*see* **Note 55**), taking care not to tear it, cover it with saran-wrap and expose to a phosphorimager screen overnight. Scan the screen with a phosphorimager.

A representative example of the analysis performed for *A. thaliana* DIS3 protein is presented in **Fig. 4a**. Control experiments with RNA substrates labeled in different ways as well as with the isolated PIN domain of DIS3 (displaying endoribonucleolytic activity) are presented in **Fig. 4b**. *[Note to Publisher: Figure 4 near here].*

3.9 XRN1 5'-3' exoribonuclease activity assay employing RNA oligonucleotide substrates with different 5'-end phosphorylation status and labeled at the 3'-end with a fluorescent dye

To assess the succeptibility of 5'-3' exoribonucleases to the phosphorylation status *in vitro*, one requires RNA substrates which have the same nucleotide sequence but bear different moieties at their 5'-ends. Uniformly labeled substrates bearing a 5'-triphosphate (5'-PPP) may be obtained by *in vitro* transcription performed in the presence of radioactive UTP (section 3.5.1). Substrates equipped with a monophosphate (5'-P), hydroxyl group (5'-OH) or methylated cap analog (5'-m^7Gppp) may be synthesized with the same protocol, by adding GMP, guanosine, or cap analog, respectively, to the reaction mixture in a molar excess over other ribonucleoside triphosphates (NTPs). Alternatively, they may be purchased as synthetic oligoribonucleotides together with a fluorescent dye, such as fluorescein (FAM),

at their 3'-end. Note that such fluoro-labeled oligonucleotides may also replace ^{32}P-labeled substrates in a number of the assays presented herein, thereby offering grater safety (handling radiochemicals requires great care) and longer substrate life (half-life of ^{32}P is 14.3 days). However, fluorescent dyes are bulky, hydrophobic moieties and sometimes interfere with enzymatic activities. The protocol presented below utilizes commercially available XRN1 enzyme and different fluorescently labeled oligoribonucleotides.

1. Prepare a 20% denaturing polyacrylamide gel using 1 mm-thick spacers and a comb (*see* **Note 18**).
2. Prepare a working solution of each fluorescently labeled RNA substrate by diluting 1 µL of the 200 µM stock to 1 µM final concentration with sterile RNase-free water (*see* **Note 47**).
3. Prepare a working solution of the protein by diluting XRN1 in 1x NEBuffer 3 to a concentration of 0.025 U/µL (*see* **Note 47**).
4. Prepare 1.5 mL microtubes, each containing 5 µL of F-dye (*see* **Note 46**).
5. In a 1.5 mL microtube, mix 2 µL of NEBuffer 3 (*see* **Note 12**), 1 µL of protein solution (step 3), 1 µL of the RiboLock™ RNase Inhibitor, and sterile RNase-free water to 15 µL. Place on ice.
6. Add 5 µL of the RNA working solution (step 2), mix by pipetting, and transfer 5 µL into the first tube containing F-dye (t0 sample) (*see* **Note 48)**. Freeze this sample in liquid nitrogen. Incubate the remainder of the reaction mixture at 37°C (*see* **Note 49**).
7. Proceed as described in section 3.8, steps 6-10, albeit using a power of 25 W during electrophoresis and a migration of bromophenol blue to ~3 cm from the bottom edge of

the gel. A representative experiment is shown in **Fig. 5**. *[Note to Publisher: Figure 5 near here].*

4 Notes

1. The insert may be obtained by PCR amplification (using SLIC-F and SLIC- primers) of cDNA isolated/prepared from the appropriate cells. If only small amounts of insert are obtained, it is highly advisable to pre-clone it into the pCR™-Blunt II-Topo® vector (part of the Zero Blunt® Topo® PCR cloning kit from Invitrogen) before attempting to clone it into the pET28M-6xHis-SUMOTag vector (usually greatly increases the probability of successful cloning). The insert might be sequenced at this stage using standard M13 forward and reverse primers (as well as other self-designed primers) to exclude the presence of unwanted mutations.
2. 10 mM dNTP mix might be prepared from solutions of individual deoxyribonucleoside triphosphates or purchased as ready-to-use mix.
3. Prepare under the fume hood by mixing 25 mL of phenol solution saturated with 10 mM Tris-HCl pH 8.0, 24 mL of chloroform, and 1 mL of isoamyl alcohol in a 50 mL Falcon tube. Wait until phases are clearly separated (the bottom phase is the organic phase that is added to the samples). Wrap the tube with aluminium foil to protect from light and store at 4°C. Do not use once the color of the organic phase has turned yellow or pink, an indication of phenol oxidation. Always wear safety glasses, gloves and a laboratory coat when working with solutions containing phenol to avoid skin/eye burns.
4. The site-directed mutagenesis primers (MUT-F/MUT-R) should be designed to introduce an inactivating mutation of one (or more) of the conserved amino acids in the catalytic center of the examined exonuclease.

5. Always wear a mask and gloves when weighing acrylamide powder and gloves when working with unpolymerized acrylamide solution, because it is a strong neurotoxin.
6. After addition of APS, the polymerization is able to take place, even in the absence of TEMED, which only catalyzes this reaction; the polymerization speed depends on the acrylamide percentage and the temperature; when pouring 10-20% polyacrylamide gels at high ambient temperature, it is advisable to store the solution of acrylamide with added APS at 4^{o}C for a short time in order to avoid its uncontrolled polymerization; however, the solution cannot be kept for too long in a refrigerator, since it will cause precipitation of urea. If this happens, it can be counteracted by heating the mixture in a waterbath set at 37^{o}C until urea is dissolved again. Afterwards, TEMED has to be added and the gel must be poured immediately.
7. Whenever air bubbles start to appear while pouring vertical polyacrylamide gel, one may easily get rid of them by leaning the gel cassette into one direction, waiting until they migrate to the upper edge of the polyacrylamide solution poured between the glass plates and knocking the front glass plate with finger knuckles until they disappear.
8. Since the volume of the primer solution mixed with F-dye to be loaded into the wells of the polyacrylamide gel is quite high, it is advisable to use a comb with wide teeth (1.5-2 cm).
9. We routinely use *E. coli* BL21-CodonPlus-RIL strain for heterologous expression of eukaryotic proteins, although other appropriate expression strains may also be used.
10. An alternative method for efficient disruption of bacterial cells may be employed if preferred, such as a French press or a microfluidizer.
11. Such a plasmid is easily constructed by subcloning a 100-500 nt-long DNA fragment derived from virtually any open reading frame into a plasmid containing either a T7 or

SP6 transcription start site upstream from the cloning site and a single-cut restriction site downstream. The restriction site is subsequently used to linearize the plasmid.

12. There are no universal buffer conditions for every exonuclease; we present the buffer composition that worked successfully in our hands for DIS3 protein homologs from different species as an example; reaction conditions, including reaction buffer, temperature and time of incubation must be optimized by performing several consecutive preliminary assays. Buffer parameters that may be tested are buffering agent (Tris, Hepes, MOPS, etc.), monovalent salt (NaCl or KCl), salt concentration, divalent cation cofactor (Mg^{2+}, Mn^{2+}, Zn^{2+}), concentration of the metal cofactor, pH, presence of the reducing agent, BSA, glycerol, *etc*.
13. We use synthetic ss17-A_x RNA as RNA oligonucleotide 1, where ss17 represents a 17 nt-long random sequence (5'-CCCCACCACCAUCACUU-3') and A_x corresponds to an oligo(A) tail of various length, ranging from 2 to 34 adenosines.
14. We use 17 nt-long compl RNA (5'-AAGUGAUGGUGGUGGGG-3') as RNA oligonucleotide 2. It is fully complementary to the 5' part of ss17-A_x RNA oligonucleotide 1 (*see* **Note 13**).
15. The comb and size of the gel depends on number of samples/conditions tested in a single assay.
16. Oligonucleotide sequence may be modified according to preferences.
17. PAGE purification may be performed as described in section 3.6.
18. Resolution is significantly better when utilizing fluorescently-labeled RNA oligonucleotides, when compared to the substrates labeled with radioisotopes. Shorter gels (we use 20 cm x 5 cm plates and 50-well comb) may be used in such cases.
19. These PCR conditions have worked satisfactorily for most inserts. However, if small amounts of the amplicon are obtained or unspecific PCR products are evident, one may

want to try standard troubleshooting strategies, such as annealing temperature gradient, attempting “touch-down” PCR protocol (where the annealing temperature is set relatively high during initial cycles of amplification and is decreased in increments in every subsequent cycle), optimizing magnesium, primer, or DMSO concentration. Ordering new primers with extended region complementary to the insert may also be taken into consideration.

20. Although 15 s of elongation during PCR amplification with Phusion polymerase might be sufficient for some plasmid templates, we find that extending this step to 30 s increases the amounts of the desired product in numerous cases.
21. Wear gloves when working with ethidium bromide solutions, since it is a strong mutagen.
22. Do not dry the pellet for too long; once overdried, it might be difficult to dissolve.
23. The preparative agarose gel can be also stained with ethidium bromide, however we prefer to use GelGreen™ dye since its fluorescent signal might be excited with blue light instead of UV, which reduces the risk of introducing mutations during excision of the gel fragments containing bands of interest.
24. In our opinion this method of estimating DNA concentration following column-based purification of DNA gives more accurate results than a spectrophotometer as column kit contaminants often absorb at 260 nm.
25. This annealing step might be also performed at 37°C for 30 minutes.
26. The mixture might be stored at -20°C for later use.
27. Other *E. coli* strains dedicated to cloning, such as DH5α, may also be used. The strain must have an active deoxyadenosine methylase (*dam*$^+$) for purposes of the site-directed mutagenesis (*see* **Note 36**). If transformation efficiency is low, electroporation of

appropriate electro-competent cells may be performed provided that the SLIC reaction mixture has been subjected to desalting by ethanol precipitation or dialysis.

28. On 10% denaturing polyacrylamide gels, the upper (xylene cyanol) and bottom (bromophenol blue) dyes migrate, respectively, as ~55 nt- and ~12 nt-long single-stranded DNA. Conditions provided in the protocol are usually sufficient to resolve the full-length primers (50-60 nt) from shorter by-products.
29. To increase the probability that the gel remains attached to only one glass plate while disassembling the gel cassette, one of the glass plates may be wiped with Rain-X before pouring the gel.
30. Usually, signal for the full-length oligonucleotide is strong enough to be distinguished from ones of by-products. Nonetheless, UV shadows can be increased by placing a TLC plate or an X-ray intensifying screen under the gel.
31. Seal the tubes with parafilm to prevent them from accidental opening and stick them in horizontal position to the platform of an incubation shaker with adhesive tape.
32. Since the elution buffer contains ammonium acetate at high concentration, addition of salt during precipitation step is not necessary.
33. A regular spectrophotometer may also be used, although with a microvolume instrument a small sample aliquot is used for measurement and then discarded, thereby avoiding risk of contaminations.
34. These amplification conditions work in most cases, although optimization may be considered if the full-length product is barely visible by agarose gel electrophoresis.
35. A distinct band of the size corresponding to the full-length plasmid should be observed; if this is not the case, the products of amplification may be processed further, but from our experience it is clear that the probability of successful mutagenesis is much lower;

however, there were cases when the mutagenesis went off well even if a smear of heterogeneous products was observed following amplification.

36. This step is essential in order to get rid of the template plasmid containing wild-type insert; otherwise, identification of the clones bearing mutated plasmid in subsequent steps of the procedure might be severely hampered by strong background. *Dpn*I is a methylation-sensitive restriction endonuclease, which means that it will digest only the original, un-mutated template, which contains methylated adenines within GATC motifs (provided that the plasmid was isolated from *dam*$^{+}$ *E. coli* strain), but not the products of mutagenesis, synthesized during PCR.
37. Verification of the presence of the restriction site introduced by the primers for mutagenesis can be quite problematic if the site is only 4 nucleotides long; in such case, it is recommended to amplify the insert (for instance using SLIC-F/SLIC-R pair of primers) from step 1 in section 3.1.1 (or even its fragment encompassing the site of mutagenesis) by PCR and then subject it to digestion with the 4-cutter enzyme. A similar control should be performed with un-mutated plasmid.
38. We prefer LB Broth Base from Invitrogen to prepare medium for the bacterial culture used for protein expression.
39. Performing induction of protein expression at a lower than physiological temperature reduces the risk of the formation of insoluble protein aggregates called inclusion bodies. In such cases, decreasing IPTG concentration and/or duration of induction may help. If the problem persists, one may use autoinduction media ***(23)***.
40. Small samples of the culture may be collected before and after induction and analyzed by SDS-PAGE to determine if induction of protein expression worked well (either by

direct staining of the gel with Coomassie Brilliant Blue or by performing western-blot detection of the protein using anti-6xHis antibodies). See -/+I samples in **Fig. 2b**.

41. We utilize an ÄKTApurifier FPLC system since it allows convenient, semi-automated purification of the protein. However, if the user does not have access to such an equipment, the purification can be done entirely manually at 4^{o}C by batch incubation of the native protein extract with a Ni-NTA agarose slurry, followed by the transfer of the resin with the bound proteins in an empty, gravity-flow chromatography column.
42. SUMO protease may be obtained from commercial sources. However, to reduce the costs of purification, we routinely prepare a home-made version of the enzyme with 6xHis-tag.
43. The column from section 3.4, step 6 can be reused here.
44. If the protein purity is not satisfactory at this stage, an additional purification method might be employed, such as ion-exchange chromatography.
45. Protein concentration may be estimated from gel band intensity (and densitometry) after running an aliquot of purified exonuclease on a SDS-PAGE gel alongside with different BSA or lysozyme dilutions of known concentrations (containing protein amounts ranging from 0.125 to 10 µg). Alternatively, a spectroscopic method (e.g., Bradford assay) may be used, albeit with a lower accuracy.
46. Total number of microtubes will depend on how many different reaction conditions and time points will be analyzed per assay.
47. The optimal molar protein:substrate ratio depends on the exoribonuclease under study. The amounts of the enzymes and RNA provided here proved adequate for a number of specific proteins and substrates but may not suit all cases.

48. When working with multiple samples in a single assay, it is most convenient to start each reaction separately by addition of the RNA substrate in time intervals (not shorter than 20 seconds between two consecutive samples, to have time for transferring the aliquot of the sample into the tube with stopping EDTA solution).
49. Temperature is another reaction parameter which must be optimized for each particular exonuclease.
50. If the phosphorimager used for TLC analysis is not sufficiently sensitive, steps 6-7 may be repeated several time to increase the amount of radioactivity per spot, provided that no more than 1 µL of sample is applied per spot every time and that all spots are allowed to dry completely before another sample application.
51. It is not absolutely necessary to use a dedicated chromatography chamber. Instead, one may use a container (*i.e.* beaker or jar) tightly sealed with parafilm or aluminum foil to ensure saturation of the inside atmosphere with the developing solution.
52. This can be accelerated by using a hair-dryer or other type of blower dryer, set at low heating temperature.
53. If the activity of the exonuclease mutant is only diminished (as for hDIS3 A507P in **Fig. 3**), the difference with wild-type activity may be better visualized at early than late time points and the reaction time course may need to be adjusted accordingly.
54. There is no universal RNA sequence to probe exonuclease activity and it is advisable to test several substrates including, for example, generic ones (with random sequence), polyribonucleotides (i.e. A_{20} or U_{20}; particularly for exonucleases which are predicted to display some sequence preferences, such as deadenylases), or ones with mixed features (we routinely use single-stranded RNAs composed of 17-mer generic sequence followed by oligo(A) tails of different lengths, ranging from 2 nt to 34 nt).

55. Using this method, it is not necessary to place the gel on the Whatman 3 mm paper and to dry it completely in a vacuum dryer before the exposure.
56. Labeled pCp may be purchased instead of being synthesized with the method that we describe. However, as it is not used as frequently as other radiochemicals, such as for instance [γ-^{32}P]-ATP, we often prepare it ourselves as presented herein. It should be noted, however, that the efficiency of 3' RNA labeling is significantly higher when using commercial rather than home-made radiolabeled pCp.
57. Circularized RNA oligonucleotide migrates slightly slower in denaturing polyacrylamide gels than the linear oligoribonucleotide of the same sequence. In practice, linear and circular RNA substrates are often prepared in parallel and purified on the same gel. In this case, it is advisable to use a 20% (instead of 10%) polyacrylamide gel to better visualize migration differences (this also facilitate separation of the circularized products from traces of linear substrates).
58. We include description of the partially double-stranded RNA substrate preparation, as some exoribonucleases (including DIS3) are active towards such substrates. Other enzymes may even prefer structured RNA molecules more than single-stranded substrates.
59. There must be no visible traces of the single-stranded substrate in the lane corresponding to the sample which was subjected to annealing; if this is the case, the amount of the RNA oligonucleotide 2 present in the annealing reaction should be increased.
60. This will give a 1:20 molar ratio of protein:substrate which we found to be optimal for assays employing DIS3 proteins and single-stranded substrates. We emphasize that this

ratio may not be appropriate for other exoribonucleases and should be optimized experimentally.

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Figure legends

Figure 1: Possible degradation patterns of RNA oligonucleotide substrates labeled in different ways as a tool for determination of exoribonuclease directionality and discriminating between exonucleolytic and endonucleolytic activities. (**a**) 5'-3' and (**b**) 3'-5' exoribonucleases may be distinguished based on products obtained with linear 5'- and 3'-end labeled substrates, unless in both cases mononucleotides are produced, which requires an additional experiment employing a special substrate (**e**). Enzymes belonging to these two groups do not have the ability to cleave circularized RNA molecules. Indeed, it is a unique feature of endoribonucleases, which may cleave RNA at a specific site (**c**) or cut it without any particular substrate specificity (**d**).

Figure 2: Purification of the N-terminally 6xHis-SUMO-tagged exonuclease following its heterologous overexpression in bacteria. (**a**) Scheme of the purification procedure. (**b**) A representative SDS-PAGE gel illustrating purification of the full-length *Arabidopsis thaliana* DIS3 (AtDIS3) protein. Samples were collected at various stages of purification, as described in the main text. A strong band at the expected size in the +I sample (following IPTG induction) indicates that protein expression was successful. The same band (although considerably weaker) was also present in lane EX, meaning that the protein is soluble, at least partially. The 6xHis-SUMO-tagged exonuclease binds to the Ni-NTA resin and is not present in flow-through (lane FT1) but is eluted in presence of high concentration of imidazole (lane EL1). Following removal of the 6xHis-SUMO epitope by the SUMO protease, the molecular mass of the protein is reduced (lane DIAL). Without 6xHis-SUMOTag, the exonuclease no longer binds to the Ni-NTA resin and is retrieved in flow-through (lane FT2)

rather than in fractions eluted in the presence of high concentration of imidazole (lane EL2). The latter fractions contain free 6xHis-SUMOTag as well as SUMO protease.

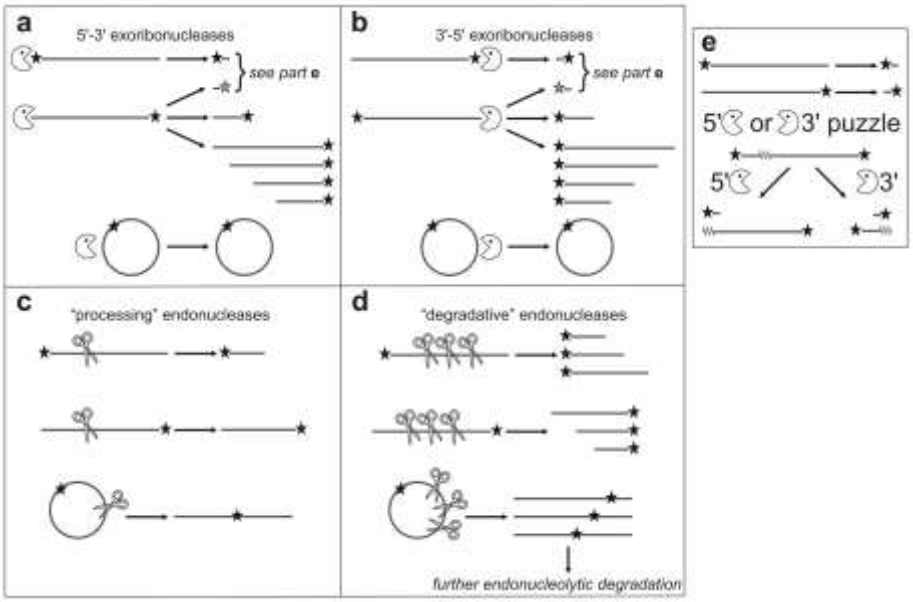
Figure 3: TLC analysis of exoribonucleolytic activity. Recombinant wild-type (WT) and D487N and A507P mutants of human DIS3 (hDIS3) were analyzed.

Figure 4: Gel-based analysis of AtDIS3 nucleolytic activities. Positions of bands corresponding to starting substrates are indicated by arrows. (**a**) AtDIS3 displays 3'-5' exoribonucleolytic activity. Recombinant WT and D489N catalytic mutant of AtDIS3 were analyzed. Final degradation products (4-5-nt) are observed only in the case of WT AtDIS3. (**b**) The N-terminal PIN domain of AtDIS3 has an endoribonucleolytic activity. The pattern of degradation products observed in the case of 5'-end labeled substrate is entirely different from that observed in pannel **a**. Moreover, ladders of products generated during degradation of 5'-end labeled and 3'-end labeled linear substrates, as well as the ability to linearize circular RNA molecule (which is further degraded by the PIN WT variant) are evidence of "degradative" endoribonucleolytic activity (**Fig. 1**).

Figure 5: Gel-based assay demonstrating dependence of XRN1 5'-3' exoribonuclease activity on the status of the substrate 5'-end. Four RNA oligonucleotides having the same nucleotide sequence, but differing by the moiety present at the 5'-terminus (monophosphate, triphosphate, hydroxyl or methylated cap) and labeled at the 3'-end with FAM were incubated with or without XRN1 protein. Positions of the substrates and short

oligoribonucleotide degradation products are marked with different arrows. Only 5'-monophosphorylated substrate is degraded efficiently by XRN1.

Figure 1.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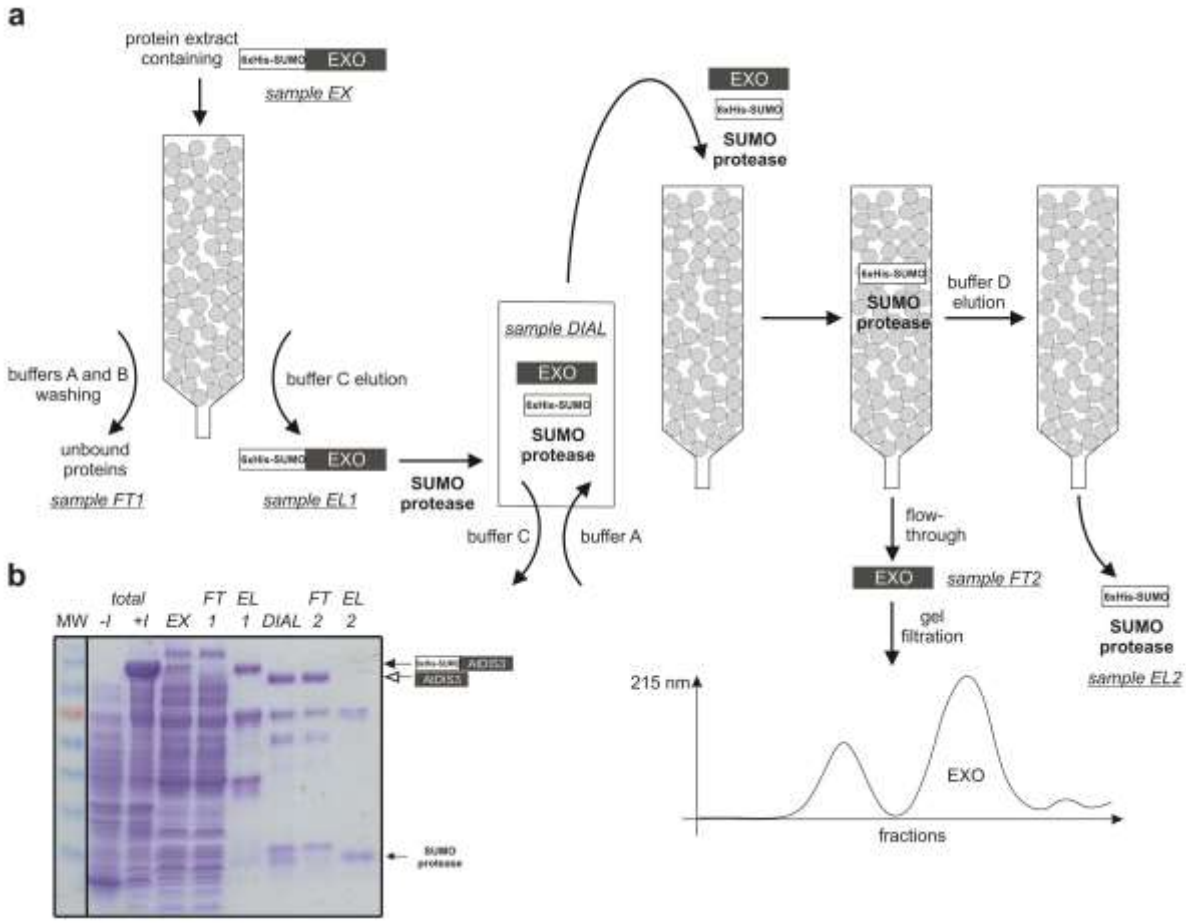
5'-3' exoribonucleases
see part e
b
3'-5' exoribonucleases
see part e
e
5' or 3' puzzle
5'
3'
c
"processing" endonucleases
d
"degradative" endonucleases
further endonucleolytic degradation

Figure 2.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EXO
sample EX
buffers A and B washing
unbound proteins
sample FT1
buffer C elution
6xHis-SUMO
EXO
sample EL1
SUMO protease
sample DIAL
EXO
6xHis-SUMO
SUMO protease
buffer C
buffer A
EXO
6xHis-SUMO
SUMO protease
6xHis-SUMO
SUMO protease
buffer D elution
flow-through
EXO
sample FT2
gel filtration
6xHis-SUMO
SUMO protease
sample EL2
215 nm
EXO
fractions
b
MW
total -I
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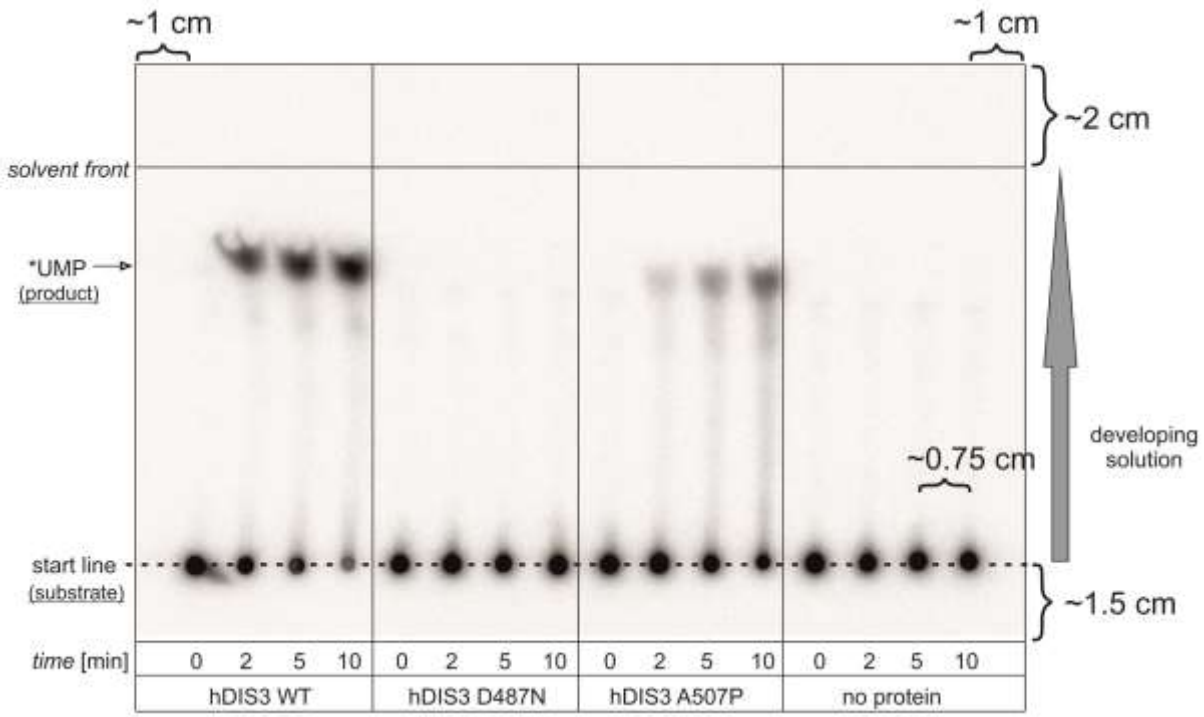
EL 2
6xHis-SUMO ADSS
ADSS
SUMO protease

Figure 3.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~1 cm
~2 cm
solvent front
*UMP
(product)
~0.75 cm
developing
solution
start line
(substrate)
~1.5 cm
time [min]
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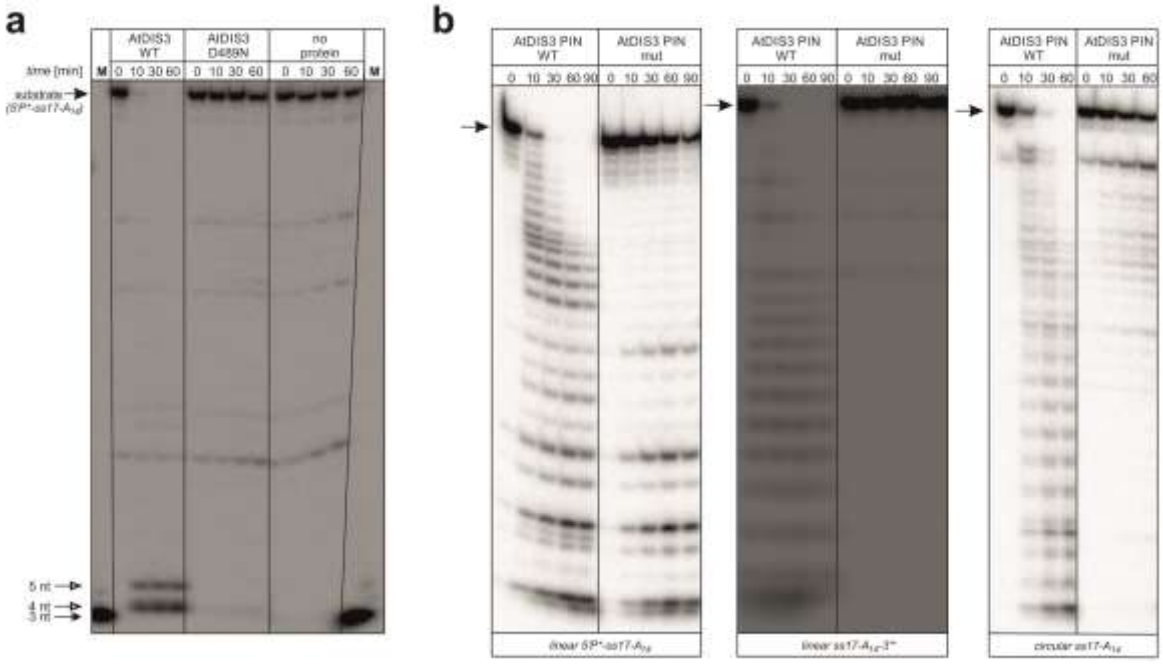
hDIS3 WT
hDIS3 D487N
hDIS3 A507P
no protein

Figure 4.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AtDIS3 WT
AtDIS3 D488N
no protein
time [min]
M
0 10 30 60
substrate
5 nt
4 nt
3 nt
b
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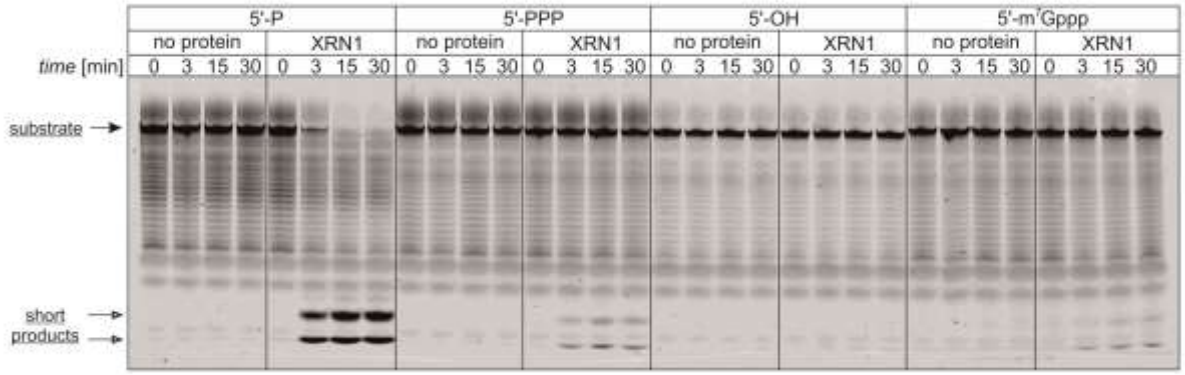
AtDIS3 PIN mut
0 10 30 60 90
linear 5P*-ss17-A14
linear ss17-A14-3*
circular ss17-A14

Figure 5.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substrate
short
products