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In Vitro Analysis of RNA Degradation Catalyzed by Deadenylase Enzymes

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Summary

In this chapter, we describe a method for purification and analysis of the enzymatic activity of deadenylase enzymes. Nearly all eukaryotic messenger RNAs are modified at the 3' end by addition of an adenosine polymer: the poly-adenosine tail. The poly(A) tail plays a central role in protein expression and mRNA fate. The poly(A) tail promotes translation of the mRNA. Shortening of the poly(A) tail, referred to as deadenylation, reduces protein synthesis and initiates destruction of the mRNA. A specialized class of exoribonucleases, called deadenylase enzymes, carries out this process. Deadenylases are found throughout eukarya but their functions remain largely unexplored. We present a detailed protocol to analyze deadenylase activity *in vitro*. First, recombinant deadenylase enzyme is over-expressed and purified from bacteria. Next, labeled RNA substrate is prepared. Deadenylation reactions are performed and reaction products are analyzed by denaturing gel electrophoresis. Reaction rates are then determined quantitatively. Crucial controls and experimental parameters are described along with practical tips that promote success.

Keywords

Poly-adenosine tail; deadenylase; exoribonuclease; RNA degradation

1. Introduction

In eukaryotes, newly synthesized mRNAs (with the exception of replication dependent histone mRNAs) are modified by the addition of a poly-Adenosine tail to the 3' end of the message [1]. The cleavage and poly-adenylation machinery recognizes sequence elements in the nascent mRNA and cleaves the mRNA at a specific site [2]. Subsequently, poly(A) polymerase adds adenosine residues to the 3' end of the mRNA [2]. Poly-adenosine tails differ in length depending on the species. For instance, in *S. cerevisiae* the nascent tails are 60–80 nucleotides, whereas mammalian tails are 200–250 nucleotides. After the addition of a 5' cap structure, poly-adenylation, and splicing of introns, the mRNA is transported to the cytoplasm where it can be translated. Throughout these events, the mRNA is susceptible to

degradation by specialized and highly regulated mRNA decay pathways [3, 4]. Messenger RNAs are often bound by regulatory factors that modulate their decay rates and translation efficiency. Quality control mechanisms also scrutinize mRNAs and destroy aberrant, defective mRNAs.

Decay of mRNAs is typically initiated by enzymatic removal of the poly(A) tail by deadenylases [3]. These enzymes are exoribonucleases that act in a 3' to 5' direction on the tail, releasing 5' AMP as a product [5–7]. Deadenylases are hydrolytic, using enzyme-bound divalent cations as essential cofactors that are coordinated by acidic residues in their active sites [3, 4]. Two groups of deadenylases have been identified based on sequence relationships of their catalytic domains [3]. The DEDD family of deadenylases, so-called because of conserved catalytic Aspartate and Glutamate residues, are related to founding members Pan2 and PARN [3, 8, 9]. This group shares similarity to the bacterial RNase D exoribonuclease [4]. The second family, EEP deadenylases, are related to a superfamily of Exonucleases, Endonucleases, and Phosphatases [3, 10, 11]. The yeast Ccr4 enzyme is the founding member of this class [12, 13].

The number of predicted deadenylases varies among eukaryotes [3, 11, 14]. Many of these enzymes have not been studied and the methods described in this chapter provide a direct, quantitative assay for measuring deadenylase catalytic activity, substrate specificity, and cofactor requirements. *In vivo*, deadenylation rates vary dramatically, possibly due to action of specific deadenylase enzymes [5, 6, 15–18]. Deadenylation is also controlled by *cis*-acting regulatory sequences and *trans*-acting factors including RNA binding proteins and small regulatory RNAs called micro-RNAs [3, 19–27]. The methods described here provide a means to study regulation of deadenylase activity in a defined biochemical system.

To begin, the deadenylase of interest must be cloned into the appropriate expression vector with an affinity tag. Multiple affinity tag purification systems have been successfully used to purify deadenylase enzymes. No single tag works best for all proteins; therefore, we recommend starting with several tags and testing protein induction and purification. Choose the tag that gives the highest yield and purity. We have successfully used Glutathione S-Transferase (GST), Halotag, and Maltose Binding Protein (MBP) tags with excellent results [26–29]. We recommend avoiding 6-histidine tags because the metal ions used on the affinity resin (usually Nickel) can inhibit deadenylase activity (our unpublished data). Further, contaminating bacterial nucleases are prone to co-elute from the metal affinity resin. Removal of the affinity tag from the deadenylase protein by engineering a site specific protease cleavage site between tag and deadenylase is often desirable. We have used Tobacco Etch Virus (TEV) protease with excellent results.

In this chapter we first describe a method to purify recombinant deadenylase enzyme: it is over-expressed as a fusion to Maltose Binding Protein in *E. coli* using conditions optimized to remove bacterial contaminants. Next, a method for preparing radioactively labeled RNA substrates is described. Finally, we describe the procedure for quantitatively measuring deadenylation activity and present instructions and suggestions for data analysis and interpretation, as well as determination of reaction rates.

2. Materials

The method described herein is by design a highly sensitive ribonuclease assay, therefore, the main contributor to experimental problems is RNase contamination. Because bacteria have abundant RNases, this is the most common and likely source of contamination. Reagents, equipment, and the scientists themselves are also possible sources of RNases.

One crucial challenge that must be met to successfully perform this analysis is to prevent RNA degradation by contaminating RNases. Chemicals must be handled only with gloves and measured with sterile, RNase free plasticware. All buffers should be prepared using sterile, RNase free, distilled deionized water. We use water purified with a Millipore Advantage A10 system with a BioPak point of use filter. All buffers should be prepared in baked glassware or RNase free disposable plasticware. A dedicated set of pipettes should be reserved for working with RNA and aerosol barrier filter pipette tips must be used. RNase Inhibitor (RNaseIn) can be added to deadenylation reactions to specifically inhibit the RNase A family, which are common environmental contaminants. Before beginning this assay, new users should consult instructions on creating and maintaining an RNase free environment. For additional information and tips for working with RNA, refer to RNA: A Laboratory Manual [30].

2.1. Protein Purification Components

1. Distilled, deionized sterile H₂O (ddH₂O), autoclaved.
2. Protease deficient *E. coli* strain transformed with deadenylase expression vector. We use BL21 Gold (Invitrogen) or KRX (Promega) strains, unless a specialized strain is required.
3. 2xYT broth with 2 mM MgSO₄. Autoclave and then add sterile glucose to a final percentage of 0.2%.
4. 40% Glucose: Dissolve 40 g into 100 mL of ddH₂O, filter sterilize.
5. Amylose affinity resin (New England Biolabs): supplied as a 50% slurry.
6. 1 M Isopropyl β -D -thiogalactoside (IPTG): Dissolve in ddH₂O, filter sterilize, store at -20°C.
7. 1 M Dithiothreitol (DTT): Dissolve in ddH₂O, store at -20°C.
8. Bugwash: 50 mM Tris-HCl pH 8.0, 10% sucrose. Filter sterilize and store at 4°C.
9. Binding buffer: 50 mM Tris-HCl pH 8.0, 2 mM MgCl₂, 500 mM NaCl. Store at 4°C. NP-40/IGEPAL at a final concentration of 0.5 % and DTT at a final concentration of 1 mM should be added immediately before use (see Note 1).
10. Protease inhibitor cocktail (50X): 50 mM phenyl methyl sulfonyl fluoride, 500 μ g/ml aprotinin, 500 μ g/ml pepstatin, 500 μ g/ml leupeptin, in 100% ethanol (see Note 2). Store at -20°C.

¹Detergent is easier to pipette accurately if it is first heated to reduce viscosity. If desired, immerse detergent bottle in a warm water bath for a few minutes before use.

11. Lysozyme: 10 mg/mL in 50% glycerol, store at -20°C
12. DNase I (from Bovine pancreas, grade II): 10 mg/mL in 50% glycerol, store at -20°C .
13. Wash buffer: 50 mM Tris-HCl pH 8.0, 2 mM MgCl_2 , and 1000 mM NaCl. Store at 4°C . NP-40/IGEPAL should be added to a final of 1% and DTT at a final concentration of 1 mM should be added immediately before use.
14. Bio-Rad econo-pac plastic chromatography columns
15. Bio-Rad micro bio-spin columns
16. Deadenylation buffer: 50 mM Tris-HCl pH 8.0, 1 mM MgCl_2 , 50 mM NaCl, and 20 % glycerol. Filter sterilize and store at 4°C . Add 1 mM DTT, final concentration, just before use.
17. Maltose elution buffer: 10 mM maltose in deadenylation buffer with 1 mM DTT. Make fresh just prior to elution.
18. Pierce 660 protein assay reagent and protein standards.
19. SDS-PAGE Loading Dye (5 $\times$): 80 mM Tris-HCl pH 6.8, 10% Glycerol, 2% SDS, 100 mM DTT, 100 mM Beta-mercaptoethanol, 0.02% Bromophenol Blue
20. SDS-PAGE running buffer (1 $\times$): 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3
21. SDS-PAGE gel: precast 4–20% gradient gels from Bio-Rad
22. SDS-PAGE Protein Ladder: PageRuler unstained protein ladder (Pierce) or equivalent.
23. Destain: 40.0% methanol and 10.0% acetic acid in water.
24. Coomassie Stain: Final concentration of 0.25% Coomassie Blue dissolved in destain and filtered.

2.2. RNA Labeling Components

Design of the substrate RNA involves consideration of several variables including length, sequence content, and method of detection. Our standard substrate is poly(A_{20}). Radioactively labeled RNA has several advantages: it is highly sensitive, versatile, and many substrates can be labeled at low cost. Once labeled, the RNA has a limited shelf-life before degradation by radiolysis. Use of radiation requires institutional approval, proper safety equipment, monitoring, and waste disposal. Additional methods of producing labeled RNA substrates have been described elsewhere [27, 30].

1. Chemically synthesized poly(A_{20}) substrate RNAs (Thermo Scientific Dharmacon or Integrated DNA Technologies; (see Note 3): 100 pmol/ μl (100 μM) stock RNA in ddH_2O . Store at -80°C .

²Do not use protease inhibitor cocktails with chelators such as EDTA as they can inactivate deadenylases. Also be aware that some protease inhibitors are toxic.

³Polyacrylamide Gel Electrophoresis (PAGE) purification is recommended for long substrates. Consult with manufacturer when ordering substrates.

1. Radioactive γ -³²P ATP: 6000 Curie/mmol, 150 mCurie/mL, 1.7 μ M (Perkin-Elmer)
2. T4 polynucleotide kinase
3. 10 $\times$ T4 kinase buffer supplied with T4 polynucleotide kinase
4. G25 sephadex: 1:1 slurry in ddH₂O. Autoclave to sterilize, store at 4°C.

2.3. Deadenylation Assay Components

1. Deadenylation Buffer: 50 mM Tris pH 8, 1 mM MgCl₂, 50 mM NaCl, 20% Glycerol (see Note 4)
2. RNA substrate diluted in deadenylation buffer
3. Purified deadenylase in deadenylation buffer (see Note 5)
4. Recombinant RNase Inhibitor (RNasin)
5. 10 $\times$ TBE Buffer: Dissolve 108 g Tris Base, 55 g Boric Acid into ddH₂O and add 20 mL 0.5 M EDTA pH 8.0. Add ddH₂O to a final volume of 1 liter.
6. Vertical Gel Electrophoresis Apparatus: We use the versatile adjustable system from CBS Scientific with leak proof gaskets and Teflon spacers and combs.
7. High Voltage Electrophoresis power supply
8. RNA gel loading buffer: 95% Formamide, 18 mM EDTA, and 0.025% SDS, Xylene Cyanol, and Bromophenol Blue
9. Denaturing 7M Urea, polyacrylamide gel (15 cm $\times$ 25 cm $\times$ 0.5 mm): 15% Polyacrylamide 19:1 bisacrylamide (see Note 6), 7 M Urea, 1 $\times$ TBE gel mix (for 20 nucleotide RNA).
10. 10% Ammonium Persulfate (APS) in ddH₂O. Store 1 mL aliquots at -20°C
11. *N,N,N',N'*-Tetramethylethylenediamine (TEMED)

2.4 RNA ladder components

1. 50 mM NaHCO₃/Na₂CO₃, pH 9.2, 1 mM EDTA

2. Methods

3.1. Purification of deadenylase enzymes

3.1.1. Bacterial Growth and Protein Induction—

1. Transform *E. coli* cells with deadenylase expression plasmid.
2. Plate on appropriate selective media, grow at 37°C.

⁴Buffer for enzymatic reaction needs to be optimized for your specific deadenylase. Optimal concentrations of divalent metals, pH, and salt will vary from protein to protein. For previously used conditions, refer to [27].

⁵Different affinity tag purifications may affect enzymatic activity. Deadenylases may be inhibited by other metals such as Ni. For this reason we advise against a His-tag purification.

⁶The percentage of polyacrylamide will be determined by the size of the RNA substrate and products being analyzed.

3. Inoculate a 5 mL liquid culture of LB media containing appropriate antibiotic from a single bacteria colony. Agitate at 37°C overnight.
4. Dilute 1.0 mL of culture into 1 liter of 2xYT with antibiotic, 0.2% glucose, and 2 mM MgSO₄ and shake at 37°C (See Note 7)
5. When OD₆₀₀=0.9, collect 1 mL “Pre-induced” sample, pellet bacteria at 14000 rpm for 1 minute, remove supernatant, and freeze bacterial pellet for subsequent analysis. Samples taken during purification will be analyzed by SDS-PAGE.
6. Induce by adding 300 µL of 1 M IPTG (final concentration 0.3 mM IPTG) for 16 hrs at 20°C (See Note 8).
7. Collect 1 ml “Induced” sample in a 1.7 mL tube, pellet bacteria at 14000 rpm, remove supernatant, and freeze bacterial pellet for subsequent analysis.

3.1.2. Harvest Bacteria—

1. Transfer bacteria to 1 L centrifuge bottles and centrifuge at 5000 g in a swinging bucket rotor for 10 minutes.
2. Resuspend pellet in 30 mL of bugwash by vortexing and/or pipetting up and down. Transfer to oakridge centrifuge tube.
3. Spin bacteria at 8000 rpm (7600 g) for 10 min in SS34 rotor. Decant supernatant.
4. Proceed to purification or, as a stopping point, snap freeze pellet in liquid N₂ and store at –80°C.

3.1.3. Prepare Protein Extract—

1. Perform all steps at 4°C or on ice to minimize protease activity and denaturation of enzyme.
2. Rapidly thaw each pellet in 25 ml of binding buffer with 1 mM DTT and 1× protease inhibitor cocktail.
3. Thoroughly resuspend pellets by pipetting and/or vortexing the sample (See Note 9).
4. Once completely thawed, add 1.25 mL of 10 mg/mL lysozyme (final concentration of 0.5 mg/ml), mix by gently inverting tube several times and incubate at 4°C for 30 min with gentle rocking.
5. Add 140 µL of 1 M MgCl₂ (5 mM final concentration) and 26 µL of 10 mg/mL DNase I (10 µg/mL final), incubate at 4°C with rocking for 20 minutes.
6. Collect 100 µL “total” sample of cell lysate for subsequent gel analysis.

⁷If desired, more starter culture (up to the full 4 mL) may be diluted into media for faster growth.

⁸Optimal induction time and temperature may vary. We frequently test 37°C for 1–3 hours and 20°C for up to 20 hours. Long, low temperature induction can facilitate production of proteins that express poorly.

⁹If cell pellet is too large to be resuspended in 25 mL Binding Buffer, more Binding Buffer may be added. Reagents added in subsequent steps should be scaled up accordingly.

7. Centrifuge cell lysate at 20,000 rpm (48,000 g) in a SS34 rotor for 30 minutes at 4°C.
8. Transfer supernatant, the protein extract, to a fresh 50 mL conical tube. Avoid disrupting the pellet. Reserve 100 µL “protein extract” sample for subsequent gel analysis.

3.1.4. Prepare affinity resin—

1. Equilibrate amylose affinity resin by washing 0.5 mL bed volume (1.0 mL of a 50% slurry) of beads per purification in a 15 mL conical tube with 10 mL of binding buffer.
2. Collect beads by centrifugation at 1000 g for 5 minutes. Decant supernatant.
3. Repeat washing step two more times for a total of 3 washes.
4. Resuspend beads in 1.0 mL of binding buffer.

3.1.5. Affinity Purification—

1. Transfer protein extract to an ice-cold 50 ml conical tube containing the pre-washed amylose affinity resin (see Note 10).
2. Allow protein to bind to the beads for 1 hour (or longer) with end-over-end rotation at 4°C.
3. Set up econo-pac plastic chromatography column on a ring stand.
4. Working at 4°C, pour sample into column and, using gravity flow, collect the flow-through in a 50 mL conical tube. Reserve a 100 µL sample of the “flow-through” for subsequent gel analysis.
5. Wash beads by adding 25 mL of wash buffer to the column, allowing liquid to drain by gravity.
6. Wash beads two additional times, for a total of three washes.
7. Wash beads three times with 25 ml of deadenylation buffer.
8. To elute protein from beads, cap the bottom of the column and add one bed volume (0.5 mL) of freshly made maltose elution buffer. (If cleaving with TEV protease and not eluting with deadenylation buffer, see Note 11 and proceed accordingly.)
9. Suspend the beads by repeatedly pipetting up and down.
10. Transfer beads to a 1.7 ml tube and incubate at 4°C for 30 minutes with end-over-end rotation.

¹⁰For more information of MBP purification, consult New England Biolab's pMAL protein fusion purification system user manual.

¹¹It may be desirable to remove the MBP affinity tag from the deadenylase. To do so, a site specific protease cleavage site such as TEV site should be cloned between the deadenylase and MBP tag. Instead of elution with maltose, the bead-bound MBP-deadenylase would be cleaved and eluted using TEV protease. We use Invitrogen's AcTEV, which is pure and free of contaminating RNases.

11. Transfer beads and elution buffer to a micro bio-spin column placed inside a 2.0 mL microfuge tube.
12. Collect first eluate by centrifugation at 1,000 g for 5 minutes at 4°C in a microfuge tube.
13. Repeat elution twice more with maltose elution buffer, incubating 5 minutes with end-over-end rotation. Collect second and third eluates.
14. Eluates should be stored at –20°C (short term) or –80°C (> 1 month).
15. Add 0.5 mL deadenylation buffer to beads left in column, resuspend by pipetting up and down, and transfer slurry to new 1.7 mL tube. Reserve 10 µL of beads after elution for subsequent gel analysis.
16. Measure protein concentration of the eluates using the Pierce 660 protein assay.

3.1.6. Analyze samples by coomassie blue stained SDS-PAGE—

1. Prepare samples in 1× SDS PAGE loading dye. Samples include 10 µl each of Pre-induced, Induced, Total cell extract, Protein Extract, Flow-through, and Beads after elution. Prepare 1 µg of total protein of each Eluates 1–3 in 1× SDS PAGE loading dye.
2. Boil samples for 5 minutes at 95°C.
3. Set up mini-protean SDS-PAGE 4–20% gel in mini-protean electrophoresis apparatus with 1 × SDS-PAGE running buffer.
4. Load samples into wells of the SDS-PAGE gel. PageRuler unstained protein ladder should be loaded into one well.
5. Perform electrophoresis at 100 volts until bromophenol blue dye reaches the bottom of the gel.
6. Remove gel from plates and transfer to clean dish containing enough Coomassie Stain to cover the gel.
7. Incubate at room temperature on a rocking platform for at least one hour.
8. Drain the Coomassie stain from the gel, and wash the gel in Destain buffer by rocking at room temperature. Wash for up to 1 hour until the gel is translucent, leaving the blue-stained protein bands visible.
9. Assess induction and purity of the protein. Confirm that the induced and purified deadenylase fusion protein corresponds to the expected molecular weight (take into account the additional 42kDa added due to the fusion of MBP). Determine if contaminants are present. A specific band containing the deadenylase fusion protein should be observed in induced sample, but not in the pre-induced sample. The deadenylase should be observed in the total and protein extract: if not, the purified protein may be insoluble.

3.2. RNA substrate 5' end labeling

1. Dilute stock RNA (100 pmol/ μ L) to 10 pmol/ μ L in ddH₂O.
2. Assemble labeling reaction (20 μ L final volume):
 - 9 μ L ddH₂O, sterile, RNase free
 - 2 μ L 10 $\times$ T4 kinase buffer
 - 4 μ L 10 μ M RNA (40 pmol per reaction, final concentration is 2 pmol/ μ L)
 - 1 μ L γ -³²P ATP, 6000 Curie/mmol, 150 mCurie/mL, 1.7 μ M
 - 2 μ L T4 polynucleotide kinase
3. Incubate at 37°C for 1.5 hrs.
4. Add 2 μ L of 0.5 mM EDTA pH 8.0
5. Heat inactivate at 70°C for 10 minutes.
6. Remove free nucleotides and salts by size exclusion spin chromatography with G25 sephadex.
7. Add 1 mL of 1:1 slurry of G25 beads to a micro bio-spin column placed in a 2 mL microfuge tube.
8. Centrifuge at 2,000 $\times$ g for 2 minutes in a microfuge.
9. Decant flowthrough and repeat centrifugation to remove the liquid from resin.
10. Place column in a new 1.7 mL microfuge tube.
11. Pipette the kinase reaction directly to the center of G25 beads in the micro bio-spin column. Cap the top of the column.
12. Spin at 2,000 g for 2 minutes to recover labeled RNA.
13. Measure the final volume of the eluate and calculate the concentration of the labeled RNA (see Note 12).
14. Store labeled RNA at -20°C until ready to use.

3.3. RNA ladder by alkaline hydrolysis

1. Prepare three microfuge tubes containing 20 μ L of 10 nM radioactive substrate RNA in 50 mM NaHCO₃/Na₂CO₃ pH 9.2 and 1 mM EDTA.
2. Heat samples to 95°C for 2, 5, and 15 minutes to hydrolyze the RNA.
3. Place samples on ice and add equal volume of RNA gel loading buffer
4. Prior to loading the ladder on denaturing 7M urea / polyacrylamide gel, heat to 95°C for 5 minutes.

¹²This assumes efficient recovery of the labeled RNA. In practice, we typically recover greater than 95% of RNA.

3.4. Deadenylation assay

3.4.1. Deadenylation reactions—The next step is to test the purified enzymes ability to degrade the labeled substrate RNA. Here, we provide a framework for reactions and data analysis. Initial experiments should focus on titrating the enzyme and measuring activity at a single reaction time point. Subsequent experiments should be performed over a time course. A number of variables should be optimized for deadenylation reactions, including divalent metal ion concentration, pH, salt concentration, temperature, and substrate concentration [27]. The size, length, and sequence content of the substrate RNA presents additional variables. Negative controls are crucial to interpret the results and should include reactions containing RNA without enzyme, RNA with purified affinity tag (i.e. MBP alone), and RNA with catalytically inactivated, mutant enzyme. An additional negative control is to add EDTA to a reaction, before the enzyme is added, so as to chelate the divalent metals. All known deadenylases require a divalent metal ion, whereas some common contaminant ribonucleases do not [3].

1. Prepare reactions on ice. In a microfuge tube, combine the following reagents in sequential order to a final volume of 20 μ L (see Note 13):
 - Deadenylation buffer
 - RNasin: 2 Units/mL final concentration
 - 200 pmol of RNA substrate (10 nM final concentration)
 - Purified Deadenylase (0 to 1 μ M)
2. Mix reactions and collect contents at the bottom of the tube by brief centrifugation.
3. Incubate reaction at 30°C (see Note 14).
4. At each time point, terminate the reaction by addition of 20 μ L of RNA gel loading buffer.
5. Heat samples at 95°C for 5 minutes and place on ice or at –20°C until ready to run on a denaturing gel.

3.4.2. Denaturing polyacrylamide gel electrophoresis—

1. Thoroughly clean glass plates and comb with alkaline, residue-free detergent. Rinse with ddH₂O and then wipe plates with 70% ethanol and a lint-free Kimwipe.
2. Treat one side of one of the glass plates with Rain-X to assist subsequent separation of the gel from the plates.
3. Assemble plates with appropriate spacers and clamps.
4. Assemble the following gel components in a 50 mL conical tube and mix gently by inversion (Do not vortex. See Note 15):

¹³When performing a reaction progress curve with multiple time points, make a master reaction and remove an aliquot at specific time points.

¹⁴Optimum temperature for the deadenylase should be determined experimentally.

¹⁵Acrylamide will begin to harden within 10 minutes.

20 mL denaturing polyacrylamide/urea gel mix

20 μ L TEMED

200 μ L of 10% APS

5. Using a pipet-aid, transfer gel mix to the assembled plates. Fill the gel assembly to the top. Use a slow but steady flow rate and avoid introduction of bubbles into the mixture.
6. Insert the comb into the top of the gel, avoiding bubbles.
7. Allow the gel to polymerize for 30 minutes.
8. Carefully remove the comb, being cautious to not tear the fragile walls of each well.
9. Place the gel into the electrophoresis apparatus, clamping a metal heat-sink against one of the glass plates to evenly distribute heat during electrophoresis.
10. Fill the reservoirs of the electrophoresis apparatus with 1 $\times$ TBE running buffer. Be sure to remove bubbles from the top and bottom of the interface of gel and buffer.
11. Pre-run the gel at 20 mA for 30 minutes or longer to warm the gel.
12. Flush out each well thoroughly after the pre-run step and before loading samples.
13. Using a fine gauge pipette tip, load each sample and RNA ladder into wells.
14. Electrophorese samples at 20 mA until Bromophenol Blue runs at least halfway through the gel.
15. Remove the glass-plate gel assembly from the electrophoresis apparatus.
16. Gently remove the Rain-X treated plate from the gel. A plastic or metal wedge can be used to pry the plates apart. Use care so as not to distort the gel.
17. Apply 3MM Whatmann filter paper to the gel. Press gently to adhere the gel to the paper.
18. Flip the paper – gel – glass sandwich so that the paper is on the bottom.
19. Gently remove the remaining glass plate from the gel, leaving the gel adhered to the Whatmann paper.
20. Wrap the gel in plastic cling wrap (see Note 16).
21. Expose gel to a phosphor screen (see Note 17).
22. Visualize radioactive RNA bands by scanning the phosphor screen using a phosphorimager.

¹⁶If long overnight exposures are necessary to visualize radioactive RNA, the gel can be dried in a gel drier before wrapping in plastic wrap. Drying the gel will diminish potential diffusion of the RNA.

¹⁷Phosphorimager is the preferred method for visualization because of its broad range of sensitivity. X-ray film can be used as an alternative.

3.5. Data Analysis

Imaging software provided with the phosphorimager is used to visualize the reaction products and make measurements. We use ImageQuant software (GE). In reactions containing deadenylase enzyme, shortening of the substrate should be observed in a dose dependent and time dependent fashion. Controls are absolutely essential so as to avoid false positive measurements of contaminating exoribonucleases. In negative control reactions lacking enzyme, only full length substrate should be observed. If shorter products are observed, this indicates that reagents and/or equipment are contaminated with RNase. No shortened products should be observed in control reactions containing MBP tag alone or with purified deadenylase enzyme wherein the catalytic residues have been mutated. If products are observed, this indicates contamination of deadenylase purification with bacterial ribonuclease(s). Also, in control reactions containing EDTA, deadenylation should not be observed. RNA degradation in EDTA treated samples is an indication of contamination by RNase A family enzymes, which unlike deadenylases, do not require divalent metal ions.

Results should be validated using multiple independent experiments and enzyme preparations. Once the deadenylase enzyme's activity has been validated, buffer conditions should be carefully optimized to obtain maximally active enzyme. These variables include the concentration of both RNA substrate and deadenylase, divalent cofactor, salt, pH, and temperature. Substrate specificity using substrates with varying sequence content, length and poly(A) length should also be tested as well. We have observed that individual deadenylases differ in the rates of shortening and in the length of the shortest poly(A) products that form. The hydrolyzed RNA ladder should be used to determine the number of adenosines that were removed by the enzyme. Substrates with different poly(A) lengths can be tested to test whether the deadenylase has a specific requirement for poly(A) length. Competition by addition of unlabeled nucleotide polymers can also be informative for analysis of deadenylase specificity. Finally important kinetic parameters such as K_m and V_{max} can be determined by measuring reaction rates with varying substrate RNA concentrations.

The rate of deadenylation can be determined by measuring the rate of loss of the full length RNA or by the accumulation of the deadenylated product over time. Using ImageQuant TL (following the softwares instructions), measure the intensities within each band across the time course. To do so, an object rectangle is drawn over each band to be measured within a lane. Carefully consider whether to apply background subtraction parameters. We typically apply a manual background subtraction by drawing an image rectangle in an empty lane, measuring its average background intensity, and apply this to all measurements. Slight variations in sample loading per lane can be normalized by dividing the measured disappearance of substrate band (or appearance of deadenylated product band) by the total radioactive RNA present in each lane.

The methods described here can allow the scientist to identify and characterize deadenylase enzymes, discover their cofactor requirements, and optimize reaction conditions. Further, the techniques can be applied to determine the kinetic parameters of the deadenylase. Importantly, these methods can be used to identify inhibitors or activators that modulate

deadenylase activity. This system can also serve as the basis of an *in vitro* assay system for measuring the effect of trans-acting RNA binding factors that modulate deadenylase function [3, 27, 25].

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