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Poly(A) polymerase-based poly(A) length assay

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Summary

mRNA polyadenylation functions in nuclear export, translation and stability. We describe an efficient protocol designed to assess poly(A) tail length that is based on 3' tailing by yeast poly(A) polymerase and product analysis to single-nucleotide resolution by capillary electrophoresis.

Keywords

poly(A) tail length assay; adenylation; yeast poly(A) polymerase; capillary electrophoresis

1. Introduction

Assays designed to determine the length of mRNA poly(A) tail have been helpful in understanding several processes in RNA biology, including mRNA transport, translation, stability, and turnover. Recent contributions include unraveling the mechanism of miRNA-mediated control of gene expression (1), discovery of cis-acting elements that support translation despite the presence of a short poly(A) tail (2), and the identification of stable transcripts with short poly(A) tails (3).

Until recently most assays for quantifying poly(A) tail length used Northern blotting after treating RNA with RNase H + oligo(dT) to remove poly(A), or a PCR approach in which the length of the poly(A) tail was determined by PCR after hybridization with modified forms of oligo(dT) (4). These gel- and PCR-based assays were time consuming, depended on use of radioactively labeled probes and required significant optimization. Here we describe a facile method for quantifying poly(A) length that is based on using poly(A) polymerase (PAP) to tag the 3' end of the poly(A) tail. When integrated with capillary electrophoresis this assay yields information on poly(A) tail length with single-nucleotide resolution. This approach is outlined in Fig. 1. In the first step yeast PAP is used to tag the 3' end of the polyadenylated tail of the mRNA with a random polymer of 12–14 guanosine and inosine residues (5). The limited processivity of yeast PAP with these nucleotides restricts the length of polymer added, and random insertion of inosine bases prevents the formation of self-paired G-tetrad structures that may inhibit the downstream steps (6). The newly synthesized GI-tag positions a complementary primer (C10T2) that is used for cDNA synthesis to the 3'-end of the poly(A) tail. PCR amplification of cDNA using a gene-specific forward primer and GI-tag-specific C10T2 primer yields a product that on gel electrophoresis appears as a smear for long poly(A) (7) and a tight band for short poly(A) tail. Higher resolution is obtained by using fluorescently labeled primers and separating PCR products by capillary

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electrophoresis (1). Fluorescent labeling of gene-specific forward primer yields comparatively cleaner results than the labeled C10T2 primer, however, the use of fluorescently labeled C10T2 primer facilitates the analysis of multiple transcripts.

2. Materials

Prepare all the solutions in RNase-free water (*see Note 1*). Carry out all the steps in an RNase-free workspace. Follow waste disposal as per the regulations. Use sterile deionized water (resistance of 18 MΩ-cm at 25°C) for PCR and DNA electrophoresis.

2.1. GI tailing

1. RNase-free water (*see Note 1*).
2. PAP: Yeast Poly(A) polymerase (600 U/μl) (Affymetrix, *see Note 2*), store at -20°C.
3. 5× PAP buffer (Affymetrix, supplied with the enzyme *see Note 3*): 100 mM Tris-HCl pH 7.0, 3.0 mM MnCl₂, 0.1 mM EDTA, 1 mM DTT, 500 μg/mL acetylated BSA, 50% glycerol. Store at -20°C.
4. 10× GTP-ITP mix (Guanosine 5'-triphosphate and Inosine 5'-triphosphate, 5 mM each): Mix, and dilute the commercially available 100 mM GTP and ITP stocks to 5 mM each in RNase-free water (*see Note 4*). Store at -20°C in small aliquots and avoid multiple freeze-thaws.
5. Ribonuclease Inhibitor (RNaseOUT, Life Technologies) Store at -20°C. (*see Note 5*).
6. 10 mM EDTA (Ethylenediaminetetraacetic acid): Dilute 1:50 times from a 0.5 M stock of EDTA.
7. RNA cleanup column (RNA Clean & Concentrator-5, Zymo Research, *see Note 6*).

2.2. cDNA synthesis

1. C10T2 DNA primer (5' CCC CCC CCC CTT 3'): 10 μM stock. Store at -20°C.
2. Superscript II (200 U/μL, Life Technologies): Store at -20°C (*see Note 7*).
3. 5× FSB (Supplied with Superscript II, Life Technologies): 250 mM Tris-HCl, pH 8.3 at room temperature, 375 mM KCl, 15 mM MgCl₂. Store at -20°C (*see Note 8*).
4. 0.1 M DTT (Dithiothreitol, supplied with Superscript II, Life Technologies): Store at -20°C.
5. 10 mM dNTP stock: Mix of 10 mM each dNTP. Store at -20°C.

2.3. PCR

1. Taq DNA polymerase (5 U/μL, NEB) (*see Note 9*).
2. 10× ThermoPol Reaction Buffer (supplied with Taq DNA polymerase, NEB): 200 mM Tris-HCl, 100 mM (NH₄)₂SO₄, 100 mM KCl, 20 mM MgSO₄, 1% Triton X-100, pH 8.8 at 25°C.
3. Oligonucleotide primers M13RC10T2 (5' CA GGA AAC AGC TAT GAC CCC CCC CCC CTT 3') or FAM-M13RC10T2 (5' 6-FAM-CA GGA AAC AGC TAT GAC CCC CCC CCC CTT 3'), GSP-F & GSP-R: 10 μM stock each. GSP-F and -R

are transcript-specific forward and reverse primers designed upstream to the predicted polyadenylation site in the gene (Fig.1, *see Note 10*).

4. 10× dNTP stock (2 mM each): Dilute 1:4 times from the commercially available 10 mM stocks of dNTP mix in water. Store at −20°C in aliquots.
5. PCR cleanup column (DNA Clean & Concentrator™-5-Capped Columns, Zymo Research, *see Note 11*).

2.4. Electrophoresis

1. Agarose: Molecular biology-grade agarose.
2. 5× TBE: Dissolve 54 g of Tris base, 27.5 g of boric acid, and 20 mL of 0.5 M EDTA (pH 8.0) in 800 mL of water and makeup the volume to 1 L. Autoclave 15 min at 15 psi pressure on a liquid cycle and store at room temperature (*see Note 12*).
3. 0.5× TBE: Dilute 1:10 times from the 5× TBE stock in water.
4. DNA loading dye: EZ-VISION® THREE 6× DNA loading dye (Amresco LLC.) (*see Note 13*).
5. Capillary electrophoresis: ABI 3730 DNA Analyzer (Applied Biosystems, *see Note 14*).

3. Methods

Handle the reagents and carry out all the steps in an RNase/nuclease-free environment. Perform all the incubations on a PCR thermal cycler. Thaw the reagents and RNA on ice. During additions, maintain all the reactions on ice. Before each incubation, mix the reaction by gentle pipetting followed by a quick centrifugation step (12,000×g for 5 s) to pool the contents.

3.1. GI-tailing of RNA

1. In a sterile 0.2 mL PCR tube, set up the GI-tailing reaction as follows (*see Note 15* for the recommended controls):

Components	Volume (in µL)
DNA-free Total RNA (<i>see Note 16</i>)	x (up to 5 µg)
RNase-free water	14.5-x
Heat at 65°C for 5 min followed by rapid cooling on ice.	
5× PAP buffer	5.0
10× GTP-ITP mix	2.5
RNase inhibitor (RNaseOUT, 40 U/µL)	1.0
PAP (600 U/µl)	2.0

2. Mix and incubate at 37°C for 60 min.
3. Add 25 µL of RNase-free water and purify the RNA using RNA cleanup column (for example RNA Clean & Concentrator-5, Zymo Research) (*see Note 6*). At the end, the GI-tailed RNA is eluted in 10 µL of RNase-free water.

3.2. cDNA synthesis

1. In a sterile 0.2 mL PCR tube, mix 9 μ L of the GI-tailed RNA with 1 μ L of C10T2 primer (10 μ M) and heat the mix at 65°C for 5 min followed by gradual cooling to 42°C and a continuous incubation for 5 min.
2. Prepare the following reverse transcription 'RT' mix in 0.2 mL PCR tube (*see Note 17* for the recommended controls):

Components	Volume (in μ L)
5 $\times$ FSB buffer	4.0
0.1 mM DTT	2.0
10 mM dNTPs	1.0
RNase inhibitor (RNaseOUT, 40 U/ μ L)	1.0
Superscript II (200 U/ μ L)	1.0
RNase-free water	1.0

3. Transfer the 'RT' mix to the denatured RNA-primer mix at 42°C.
4. Mix, and incubate at 42°C for additional 50 min followed by heating at 94°C for 5 min.
5. After cooling to room temperature, dilute the reaction by adding 20 μ L of PCR grade water (*see Note 18*).

3.3. PCR

1. As outlined in Fig.1 and section 2.3, design and synthesize the primers using the RNA/gene sequence of interest (*see Note 10*).
2. In a sterile 0.2 mL PCR tube, set up PCR as follows (TSR=tail-specific reaction, and GSR=Gene-specific reaction) (A separate tail-specific reaction is used for the FAM-labeled primer, *see Note 19*):

Components	Volumes in μ L	
	TSR	GSR
10 $\times$ Taq polymerase buffer	5.0	5.0
10 $\times$ dNTPs	5.0	5.0
10 μ M GSP-F primer	1.0	1.0
10 μ M GSP-R primer	-	1.0
10 μ M M13RC10T2/FAM-M13RC10T2 primer	1.0	-
Diluted cDNA	2.0	2.0
Taq DNA polymerase (5 U/ μ L)	0.5	0.5
PCR grade water	to 50	to 50

3. Mix and place the tube on a thermocycler. Amplify the cDNA using the cycling conditions given in Table 1 (*see Note 20*).

3.4. Electrophoresis

A) Gel electrophoresis

1. Electrophorese appropriate size markers and 5 μ L of each reaction on a 2% agarose gel in 0.5 $\times$ TBE buffer (*see Note 13, 21*).
2. Stain the gel, if required, (*see Note 13*) and capture a gel-image using a gel documentation system (Refer Fig. 2 for a representative image).

B) Capillary electrophoresis

1. To remove unused labeled primers and their dimers, 25 μ L of the FAM-labeled TSR reaction is purified using PCR cleanup column (for example, DNA Clean & ConcentratorTM-5-Capped Columns, Zymo Research) according to the manufacturer's instructions (*see Note 11*). Elute the product with 25 μ L of water.
2. Mix 1, 1:2, 1:4, and 1:8 dilutions of the purified product with an appropriate size-standard and analyze on ABI 3730 DNA Analyzer following the standard AFLP (Amplified-fragment length polymorphism) assay (*see Note 14*). A representative electropherogram is in Fig. 3).

3.5. Data analysis

1. Analyze the gel image or data from capillary electrophoresis for the tail-specific reaction and determine the variables indicated in Step 2 below. Analysis of gel image can be performed using software, such as ImageJ (NIH), Quantity One 1-D Analysis Software (Biorad) or any other compatible software. For data from capillary electrophoresis on ABI 3730 DNA analyzer (.fsa files), we recommend using the GeneMapper[®] v4.1 package (Applied Biosystems) with the standard AFLP analysis protocol.
2. Use the following formulae to assess the length of poly(A) tail on the mRNA of interest:

Shortest poly(A) tail length= $S - D - 27$

Longest poly(A) tail length= $L - D - 27$

Length of poly(A) tail on the most abundant mRNA species= $M - D - 27$ (only applicable to Capillary electrophoresis data)

where, S and L are respective sizes of the smallest and largest PCR product in the smear or electropherogram. M is the highest peak on the electropherogram. D is the distance between the GSP forward primer and the end of the last exon or the predicted polyadenylation site in the gene sequence. It includes the length of the primer-binding site. In the formulae, number '27' is the length of the adapter in M13RC10T2 without the last two T's. All the variables are in bp or nucleotides.

4. Notes

1. To prepare RNase-free water, add 100 μ L of Diethylpyrocarbonate (DEPC) per liter of glass-distilled water. Mix until globules disappear and let stand overnight. Autoclave for 30 min at 15 psi and store in aliquots at -20°C in RNase-free tubes. Note that DEPC reacts with primary amines such as Tris, and as such commercially available concentrated solutions of Tris should be added autoclaved DEPC-treated water.
2. Use of other polymerases such as *E. coli* Poly(A) polymerase (8) is not recommended.

3. 5× PAP buffer can be made from stock reagents of higher concentrations in RNase-free water (*see Note 1*).
4. Commercially available sodium salts of GTP and ITP can also be used to make stocks solutions in RNase-free water. Do not include the GTP/ITP mix with RNA in the denaturation step. GTP is unstable at high temperatures.
5. Other commercially available RNase-inhibitors such as Superscript and RNasin may also be used.
6. Glycogen/LiCl precipitation is a cheap alternative to column-based purification; however, these procedures are susceptible to a considerable RNA loss. Chilling the contents during the precipitation step at −20°C for 1 hour improves yield.
7. Use of alternative reverse transcriptase may require optimization (*see Note 16*).
8. FSB Buffer may also be made from stock reagents in RNase-free water (*see Note 1*).
9. A change in the source of thermophilic DNA polymerase may require optimization of PCR conditions.
10. Considering the PCR mix composition, use standard parameters for designing the primers (Length=20–40 nucleotides, %GC=40–60, and melting-temperature=65–75°C). Avoid primers showing a tendency to form secondary structures and dimers. We also recommend a verification step for sequence-specificity. This may be done by a transcriptome-level comparison of primer sequences using the BLAST tool at the GenBank database. In addition, the distance between the binding-site of the forward primer and the polyadenylation-site is of considerable importance. This influences the choice of gel separation and the method of analysis. For TSP products, we recommend an amplicon size below 1000 bp including the length of the A-tail. For fluorescent labeling of TSP products, 6-FAM-labeling on GSP-F primer instead of M13RC10T2 yields much cleaner data, however, it may not be cost effective when analyzing multiple transcripts. In addition, apart from 6-FAM, the users may also use compatible fluorophores such as ROX™, TAMRA, and PET®. While designing the labeled primers, avoid labeling of bases that show proximal-base quenching effect, e.g., 6-FAM to a G base.
11. This is an optional step. A PCR mix can be directly analyzed by gel or capillary electrophoresis. PCR cleanup may be necessary to avoid interference due to salts and residual primers in capillary electrophoresis. We highly recommend the cleanup columns from Zymo Research Corporation because their design efficiently reduces carry-over residual ethanol in the eluates. Contaminating ethanol, being lighter, causes loss of PCR product during loading of wells in the gel electrophoresis step.
12. 5× TBE stock is more stable at room temperature than 10× stock and does not have the risk of precipitation from being too concentrated.
13. DNA loading dye used here pre-stains the PCR products. Users may also use other available staining methods involving ethidium bromide and non-toxic stains, such as SYBR GOLD. The fluorescently labeled TSP product may not be visible on gel due to a low cycle PCR.
14. For capillary electrophoresis, we recommend the use of ABI 3730 DNA analyzer (Applied Biosystems). The manufacturer recommends use of GeneScan Size Standards (Applied Biosystems), which are available in various size-ranges. Use

size standard that are appropriate for the expected size of the FAM-labeled TSP products.

15. The use of 'No RNA' and 'No PAP' controls is a good practice for GI tailing reaction.
16. The amount of RNA input can be empirically determined by the abundance of transcript of interest. DNase I treatment of input RNA is also highly recommended.
17. To rule out amplification from DNA contamination, we recommend a 'No RT' control for all the gene-specific reactions.
18. Quantitative PCR for housekeeping transcripts, such as actin and GAPDH, may be used to assess the efficiency of cDNA synthesis. However, amplification in "No PAP control" may be seen due to non-specific priming. We have seen a 21-fold amplification of β -Actin transcripts in 'PAP treated' over 'No PAP' control reactions ($\Delta C_q > 4$). To avoid non-specific priming, cDNA synthesis may be performed at higher temperature with a thermally stable reverse transcriptase.
19. No template control is highly recommended.
20. In certain cases, an optimization of PCR conditions may be essential, especially, the annealing temperature.
21. Percentage and choice of gel (agarose vs polyacrylamide) depend upon the size of the expected products. Note that the Bioanalyzer system (Agilent Inc.) can also be used as an alternative to gel or capillary electrophoresis.

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Figure 2. Assessment of poly(A) tail length of *in vitro* transcribed luciferase mRNAs with varying length poly(A) tails

Lanes, A20, A31, and A58 are luciferase mRNAs with the stated number of adenosines at their 3' terminus. Note the TSP lanes show smears proportional to poly(A) tail length. (Shaded wedge=TSP products, Arrow=GSP products, 'No RT' is No reverse transcriptase reaction, M=DNA ladder, & bp=base pairs).

Figure 3. Determination of β -actin mRNA poly(A) tail length by capillary electrophoresis

FAM-labeled TSP* products were separated by capillary electrophoresis and the products are shown as relative fluorescence as a function of product size. Each peak represents a single nucleotide difference in poly(A) tail length. The GSP forward primer (TTT GAA TGA TGA GCC TTC GTG CCC) is 161 nt upstream of the predicted poly(A) site. The smallest product (dark arrow) is 196 bp from GSP-F and the longest is 271 bp (gray arrow). Using the formula from **Section 3.5**, the length of the poly(A) tail on β -actin mRNA from HEK293 cells spans 8 to 83 nucleotides. For legend information refer Figure 1.

Table 1

PCR cycling conditions.

Step No.	Time/Temperature	Remark(s)
1	3 min at 95°C	Hold
2	10 s at 95°C	30 cycles for GSR and 15–20 for FAM-TSR ^a
3	30 s at 60°C ^b	
4	1 min at 72°C ^c	
5	1 min at 72°C	Hold
6	4°C	Hold

^aLow cycles ensures linearity of the reaction in the FAM-labeled TSR. The number of cycles also depends upon the expression level of the transcript.

^bAnnealing temperature may require optimization.

^cSet polymerization time to 1 min/kb.