Chapter 17

Genome-Wide Profiling of Uncapped mRNA

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Abstract

Gene transcripts are under extensive posttranscriptional regulation, including the regulation of their stability. A major route for mRNA degradation produces uncapped mRNAs, which can be generated by decapping enzymes, endonucleases, and small RNAs. Profiling uncapped mRNA molecules is important for the understanding of the transcriptome, whose composition is determined by a balance between mRNA synthesis and degradation. In this chapter, we describe a method to profile these uncapped mRNAs at the genome scale.

Key words: mRNA, Uncapping, RNA degradation, Transcriptome

1. Introduction

Widely used high-throughput transcriptome profiling approaches have been successful in dissecting gene regulatory networks. Still, transcriptome-wide profiling is often compromised by the complicated life cycle of mRNA, from transcription to decay. The different forms of RNA molecules that exist throughout the mRNA life cycle (from full-length, capped RNA to uncapped or cleaved RNA) are often indistinguishable by traditional RNA profiling. As the abundance of mRNA within cells is determined by the rates of mRNA synthesis and degradation, the reconstruction of gene expression networks clearly requires data for mRNA degradation and other modes of regulation of mRNA transcript abundance. Numerous studies indicate that mRNA degradation is a determining factor for the steady-state levels of mRNAs in cells. The decay of mRNA, in turn, can be affected by various developmental and environmental stimuli (1, 2).

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The degradation of mRNA in eukaryotes can be initiated by one of several highly conserved pathways. Usually, general mRNA decay is initiated by deadenylation via a variety of mRNA deadenylases that shorten the 3′ poly(A) tail (3). A decapping enzyme complex consisting of DCP1 and DCP2 then removes the 5′-modified guanine nucleotide cap structure. The decapped transcripts are progressively digested by a 5′–3′ exonuclease known as XRN1 in yeast and human (2). As an alternative, deadenylated mRNAs may be degraded in a 3′–5′ direction by the cytoplasmic exosome complex. In addition, nonsense-mediated mRNA decay (NMD) is a quality control system that rapidly removes mRNAs containing premature termination codons through deadenylation-independent decapping. Endonuclease cleavage can also initiate mRNA degradation, mediated either indirectly by small RNA-mediated silencing or directly by endonuclease-mediated cleavage, both of which generate uncapped mRNA. These pathways (with the exception of the 3′–5′ decay pathway) produce mRNA fragments with a free 5′ monophosphate group.

The presence of this free 5′ phosphate has been exploited to design the RNA ligase-mediated 5′-RACE to map cleavage sites of individual transcripts (4, 5). More recently, the method has been extended to the genome scale to map all uncapped mRNA molecules (6–10). Briefly, the free 5′ phosphate group of uncapped mRNA is used for the T4 RNA ligase-mediated ligation to an RNA adaptor, which is subsequently used for RNA purification and selective cDNA synthesis. The resulting cDNA can then be profiled by microarray hybridizations or high-throughput sequencing (Fig. 1).

2. Materials

1. Nuclease-free sterile water.
2. Nuclease-free sterile tubes.
3. Total RNA isolation kit or reagents, such as the RNeasy mini kit (Cat. No. 74104, Qiagen, Valencia, CA).
4. Poly(A)$^+$ RNA purification kit, such as the Micro-FastTrack 2.0 kit (Cat. No. K1520-02, Invitrogen, Carlsbad, CA).
5. RNA Adaptor: 5′-CGA CUG GAG CAC GAG GAC ACU GAC AUG GAC UGA AGG AGU AGA AA-3′.
6. T4 RNA ligase (5 U/μl) and 10× ligase buffer (Ambion).
7. Ribonuclease inhibitor, such as RNasin (40 U/μl) (Promega).
8. 3′ Biotinylated DNA probe: 5′-GTC CTC GTG CTC CAG TCG/3BioTEG/-3′.

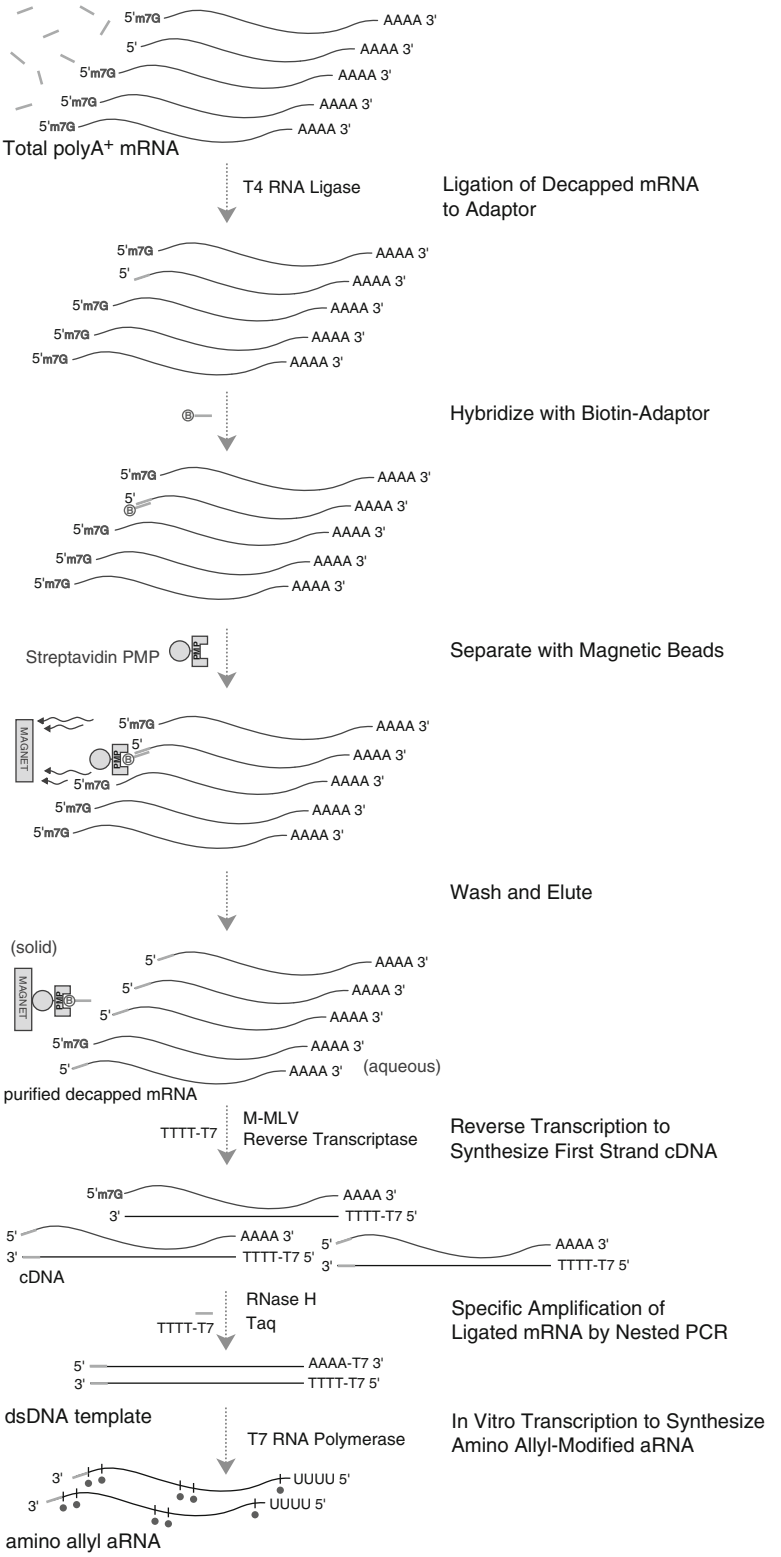

Fig. 1. Schematic description of the steps for isolation of uncapped mRNA, reverse transcription, and labeled aRNA amplification for microarray hybridization.

9. Streptavidin-paramagnetic particles (SA-PMPs) (Promega).
10. T7dT primer: 5′-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG TTT TTT TTT TTT TTT TTT TTT TTT-3′.
11. Superscript II RT (200 U/μl) (Invitrogen).
12. dNTPs, 2.5 mM each.
13. DTT, 0.1 M.
14. RNase H (2 U/μl) (Cat. No. 18021-014, Invitrogen).
15. Taq DNA polymerase, such as ExTaq (5 U/μl) (Cat. No. TAK RR001A, Clontech, Madison, WI).
16. GR primer: 5′-CGA CTG GAG CAC GAG GAC ACT GA-3′.
17. GRN primer: 5′-GGA CAC TGA CAT GGA CTG AAG GAG TA-3′.
18. PCR cleanup kit, such as DNA Clean and Concentrator (Cat. No. D4003, Zymo, Orange, CA).
19. T7 RNA polymerase, such as the Megascript T7 kit (Ambion).
20. ATP, CTP, and GTP mix, 25 mM each.
21. UTP, 50 mM.
22. Amino allyl-UTP (aa-UTP), 50 mM (Ambion).
23. 0.4 M Na_2CO_3, pH 8.5.
24. Cy3 and Cy5 mono-reactive dye (GE Healthcare).
25. 4 M hydroxylamine.
26. 10× Fragmentation reagent and stop solution (Ambion).
27. Spin-50 mini-columns.
28. T7 Oligo(dT) primer: 5′-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG TTT TTT TTT TTT TTT TTT TTT TTT-3′.

3. Methods

3.1. Extract Poly(A)$^+$ RNA

1. Isolate total RNA using the RNeasy kit, or a similar method, according to the manufacturer's instructions. At least 500 μg of total RNA is required per experiment.
2. Isolate poly(A)$^+$ RNA from the total RNA using the Micro-FastTrack 2.0 kit according to the manufacturer's instructions. This method generally yields >500 ng of poly (A)$^+$ RNA.

3.2. Adaptor Ligation

1. Vacuum dry 500 ng of poly (A)$^+$ RNA to 1 μl.
2. Assemble the adaptor ligation mix in a nuclease-free tube on ice:

Poly $(A)^+$ RNA	500 ng
RNA adaptor (0.3 μg/μl)	1 μl
10× T4 RNA ligase buffer	1 μl
T4 RNA ligase (5 U/μl)	2 μl
RNasin (40 U/μl)	1 μl

Nuclease-free water to a final volume of 10 μl.

3. Mix well and incubate reactions for 1.5 h at 37°C.

3.3. Uncapped Poly $(A)^+$ RNA Purification

1. Add 240 μl of nuclease-free water to the ligation reaction.
2. Place the tube in a 65°C heating block for 10 min.
3. Add 1.5 μl of the (50 pM/μl) 3′ biotinylated DNA probe and 6.5 μl of 20× SSC. Mix gently and incubate at room temperature until completely cooled (~10 min or less).
4. Prepare 1.2 ml of sterile 0.5× SSC by combining 30 μl of 20× SSC with 1.17 ml of nuclease-free water.
5. Prepare 1.4 ml of sterile 0.1× SSC by combining 7 μl of 20× SSC with 1.393 ml of nuclease-free water.
6. Resuspend one tube (0.6 ml) of the SA-PMPs per two isolations by gently flicking the bottom of the tube until the particles are completely dispersed, and then capture them by placing the tube in the magnetic stand until the SA-PMPs have been collected at the side of the tube (~30 s).
7. Carefully remove the supernatant while the tube sits in the magnetic stand.
8. Wash the SA-PMPs three times with 0.5× SSC (300 μl per wash), each time capturing them using the magnetic stand and carefully removing the supernatant.
9. Resuspend the washed SA-PMPs in 100 μl of 0.5× SSC and split into two tubes. Use one tube per reaction.
10. Add the entire contents of the annealing reaction (from step 3) to one tube (~50 μl) of washed SA-PMPs.
11. Incubate at room temperature for 10 min. Gently mix by inverting every 1–2 min.
12. Capture the SA-PMPs using the magnetic stand and carefully remove the supernatant without disturbing the SA-PMP pellet.
13. Wash the particles four times with 0.1× SSC (150 μl per wash) by gently flicking the bottom of the tube until all the particles are resuspended. After the final wash, remove as much of the supernatant as possible without disturbing the SA-PMPs.

14. Elute the mRNA by resuspending the final SA-PMP pellet in 50 µl of nuclease-free water (preheated to 55°C). Gently resuspend the particles by flicking the tube.
15. Magnetically capture the SA-PMPs and transfer the eluted RNA to a sterile, nuclease-free tube. Do not discard the particles.
16. Repeat the elution step by resuspending the SA-PMP pellet in 75 µl of nuclease-free water (preheated to 55°C). Repeat the capture step, pooling the eluate with the RNA eluted in the previous step.
17. Freeze the eluate at −20°C for 10 min, and then vacuum dry to reduce the volume to 7 µl.

3.4. Reverse Transcription to Synthesize First-Strand cDNA

1. Add 1 µl (100 ng/µl) of T7dT primer.
2. Incubate for 10 min at 70°C in a heating block. Centrifuge briefly.
3. Assemble the reverse transcription mix in a nuclease-free tube at RT:

5× First-strand buffer	4 µl
0.1 M DTT	2 µl
2.5 mM dNTP mix	4 µl
RNasin (40 U/µl)	1 µl
SuperScript II RT (200 U/µl)	1 µl

4. Mix well and transfer 12 µl of the mix to each RNA sample.
5. Incubate reactions for 2 h at 42°C.

3.5. Second-Strand cDNA Synthesis

1. Add 1 µl of RNase H (2 U/µl) and incubate for 15 min at 37°C, and then transfer to ice.
2. Assemble the first PCR mix in a nuclease-free tube on ice:

10× ExTaq buffer	18 µl
2.5 mM each dNTP mix	16 µl
20 µM GR primer	8 µl
T7dT primer (100 ng/µl)	8 µl
ExTaq (5 U/µl)	2 µl
Nuclease-free water	127 µl

3. Mix well and transfer 179 µl of the mix to each sample on ice. Mix well and split into four PCR tubes on ice.

4. The PCR program is (see Note 1) as follows:
   (a) 94°C for 2 min.
   (b) 94°C for 30 s.
       72°C for 3 min.
       Repeat (b) for two cycles.
   (c) 94°C for 30 s.
       70°C for 3 min.
       Repeat (c) for two cycles.
   (d) 94°C for 30 s.
       66°C for 30 s.
       68°C for 3 min.
       Repeat (d) for two cycles.
   (e) 72°C for 10 min.
5. Combine the tubes and purify with DNA Clean and Concentrator (Zymo), or a similar kit, following the manufacturer's instructions. Elute with nuclease-free water into 16 μl.
6. Assemble the second PCR mix in a nuclease-free tube on ice:

10× ExTaq buffer	5 μl
2.5 mM dNTP mix	4 μl
20 μM GRN primer	1 μl
T7dT primer (100 ng/μl)	1 μl
ExTaq (5 U/μl)	0.5 μl
Nuclease-free water	22.5 μl

7. Mix well and transfer 34 μl of the mix to each sample in a PCR tube on ice.
8. The PCR program is as follows:
   (a) 94°C for 2 min.
   (b) 94°C for 30 s.
       65°C for 30 s.
       72°C for 3 min.
       Repeat (b) for five cycles.
   (c) 72°C for 10 min.
9. Purify the PCR products with DNA Clean and Concentrator (Zymo), or a similar kit, following the manufacturer's instructions. Elute with nuclease-free water into 14 μl (see Note 2).

3.6. In Vitro Transcription to Synthesize aRNA

1. Assemble the transcription mix in a nuclease-free tube at room temperature:

10× Reaction buffer	4 μl
25 mM ATP, CTP, and GTP mix	12 μl
50 mM UTP	3 μl
50 mM aa-UTP	3 μl
T7 enzyme mix	4 μl

2. Mix well and transfer 26 μl of the mix to each sample. Mix thoroughly by pipetting up and down two to three times, and then flicking the tube three to four times, and centrifuge briefly.
3. Incubate reactions for ~14 h at 37°C in a hybridization oven.
4. Stop the reaction by adding 60 μl nuclease-free water.
5. Clean up the RNA using the RNeasy kit, or a similar kit, following the manufacturer's instructions. Elute with nuclease-free water into 100 μl.
6. Determine the concentration of each aRNA sample by measuring its absorbance at 260 nm using a spectrophotometer, such as NanoDrop 1000A (see Note 3).

3.7. Dye Coupling

1. Resuspend one Cy3 or Cy5 dye vial in 88 μl DMSO (see Note 4).
2. Place 5–20 μg of aRNA in an amber microcentrifuge tube and vacuum dry on medium or low heat until no liquid remains (see Note 5).
3. Add 7 μl of nuclease-free water and 2 μl of 0.4 M Na_2CO_3 (pH 8.5) to the dried aRNA and resuspend thoroughly by vortexing gently.
4. Add 11 μl of the corresponding dye, previously resuspended in DMSO. Mix well by vortexing gently.
5. Incubate the reaction in the dark at room temperature for 30 min.
6. Add 4.5 μl of 4 M hydroxylamine and mix well by vortexing gently.
7. Incubate the reaction in the dark at room temperature for 15 min.
8. Add 5.5 μl of nuclease-free water and bring the volume to 30 μl.
9. Clean up the labeled aRNA using RNeasy kit (Qiagen), or a similar kit, following the manufacturer's instructions. Elute with nuclease-free water into 30 μl.

10. Vacuum dry the labeled aRNA to 9 µl.
11. Add 1 µl of 10× fragmentation reagent and mix well.
12. Incubate the reaction at 70°C in a heating block for 10 min.
13. Add 1 µl of stop solution and 15 µl of nuclease-free water.
14. Prepare a Spin-50 column by spinning for 3 min at 1,000 × *g*. Transfer the column to a nuclease-free amber tube.
15. Load the entire reaction from step 12 onto the center of the Spin-50 column, and then centrifuge for 3 min at 1,000 × *g*.
16. Determine the concentration and dye incorporation rate of the aRNA sample by measuring its absorbance at 260, 550, and 650 nm using a NanoDrop 1000A spectrophotometer (see Note 6).

4. Notes

1. The indicated PCR cycling parameters are optimized for using ExTaq DNA polymerase with an MJ Research PTC-200 thermo cycler and with *Arabidopsis thaliana* RNA. The annealing temperatures may need further optimization if other conditions are used. To obtain the most suitable parameters, it is recommended to extend the PCR reaction with additional cycles and to analyze by agarose gel electrophoresis the PCR products that are obtained with different annealing temperatures.
2. Although the following steps produce labeled aRNA for microarray hybridization, direct sequencing may also be used to quantify uncapped poly $(A)^+$ RNA and to map the 5′ ends.
3. The size distribution of aRNA can be evaluated using an Agilent 2100 bioanalyzer or by denaturing agarose gel electrophoresis. The expected profile of the aRNA sample is a distribution of sizes from 250 to 5,000 nt, with the smear peaking at around 1,000–1,500 nt.
4. Cy3 and Cy5 reactive dyes are sensitive to light and water. Unused DMSO-dissolved dye should be stored at −20°C for up to 1 month and be out of moisture.
5. Check the progress of drying every 5–10 min, and do not overdry.
6. The dye incorporation rate, i.e., the number of dye molecules incorporated per 1,000 nt of labeled aRNA, can be estimated using the following formula:

$$\text{Incorporation rate} = (A_{\text{dye}}/A_{260}) \times (9,010/\text{cm/M})/ (\text{dye extinction coefficient}) \times 1,000.$$

Cy3 has an absorbance maximum at 550 nm and a dye extinction coefficient of 150,000. Cy5 has an absorbance maximum at 650 nm and a dye extinction coefficient of 250,000.

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