Chapter 6

Fractionation of Non-polyadenylated and Ribosomal-Free RNAs from Mammalian Cells

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Abstract

Most of mRNAs and well-characterized long noncoding RNAs are shaped with 5′ cap and 3′ poly(A) tail. Thereby, conventional transcriptome analysis typically involved the enrichment of poly(A)+ RNAs by oligo(dT) selection. However, accumulated lines of evidence suggest that there are many RNA transcripts processed in alternative ways, which largely failed to be detected by oligo(dT) purification. Here, we describe an enrichment strategy to purify non-polyadenylated (poly(A)−/ribo−) RNAs from total RNAs by removal of poly(A)+ RNA transcripts and ribosomal RNAs. In the combination with high-throughput sequencing methods, this strategy has been successfully applied to identify the rich repertoire of non-polyadenylated RNAs in vivo.

Key words RNA fractionation, Non-polyadenylated RNAs, Long noncoding RNAs, Deep sequencing

1 Introduction

Nascent RNA precursors undergo multiple co-/post-transcriptional processing and modification events during their maturation. The 3′-end maturation of a nascent transcript is critical for allowing the release of the RNA from the transcription template and for assuring the functionality of a mature mRNA. The consequence of polyadenylation generates up to 200–250 adenosines at 3′ end of a mRNA, which is crucial for mRNA stability, nucleocytoplasmic export, and translation [1]. 3′-Polyadenylation occurs at almost all eukaryotic mRNAs and most of well-characterized long noncoding RNAs (>200 nt in length) [2]. Thus, transcriptome analysis with tiling arrays and deep sequencing (RNA-seq) in most studies typically started with the enrichment of polyadenylated (poly(A)+) RNAs by oligo(dT) selection [3–6]. Yet, this approach precludes the detection of non-polyadenylated (poly(A)−) RNA transcripts that lack a poly(A) tail.

Besides housekeeping noncoding RNAs transcribed by RNA polymerase I and III, such as ribosomal RNAs (rRNAs) and

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other small regulatory RNAs, it is generally believed that replication-dependent histone mRNAs transcribed by RNA polymerase II were the only cellular mRNAs without 3′ poly(A) tail in eukaryotes [7]. These replication-dependent histone pre-mRNAs end in an evolutionarily conserved 3′ stem-loop structures, which direct U7 snRNA-mediated 3′-end maturation [7]. Interestingly, recent large-scale transcriptomic studies have suggested mature mRNAs with short poly(A) tails and long noncoding RNAs (lncRNAs) without a poly(A) tail in mammalian cells [8–20, 26]. For example, Cheng et al. used tiling arrays to detect polyadenylated and non-polyadenylated RNAs from ten human chromosomes and reported that non-polyadenylated transcripts comprise the major proportion of the transcriptional output of the human genome [10]. Wu et al. used 454 sequencing to characterize the 3′-end formation of transcripts and identified significant proportion of non-polyadenylated transcripts in HeLa cells [11]. Moreover, some well-characterized lncRNAs *MALAT1* and *MENβ* were also reported to be non-polyadenylated, and processed at their 3′ ends by RNase P [13, 21] to generate a stable triple helical structure [22, 23]. Furthermore, recent studies also revealed that a number of Pol II-transcribed lncRNAs originated from intergenic, enhancer or intron regions can be processed to non-polyadenylated formats in alternative ways [14–20, 24].

Search for novel non-polyadenylated RNAs was hampered by the abundance of ribosomal RNAs, which are highly expressed in cells. In this case, many studies typically performed depletion of ribosomal RNAs before large-scale transcriptomic studies [11, 12, 17, 18]. By comparing ribosome-depleted (ribo−) RNAs, which contain both poly(A)+ and poly(A)− transcripts, with oligo(dT)-selected poly(A)+ RNAs, it has led to the discovery of many new poly(A)− transcripts [12, 25]. However, ribosomal depletion only removes abundant ribosomal RNAs, but cannot physically fractionate poly(A)− RNAs from poly(A)+ transcripts, which leads to the difficulty in the direct annotation of poly(A)− RNAs. Recently, an integrated method to separate poly(A)− RNAs from both poly(A)+ RNAs and ribosomal RNAs was applied to obtain a relatively pure population of poly(A)−/ribo− RNAs for high-throughput deep sequencing [26], and further studies have revealed many previously un-annotated lncRNAs from introns in humans [16, 20]. In addition, with the similar strategy for poly(A)− RNAs preparation followed by deep sequencing analyses, additional poly(A)− lncRNAs were broadly detected in various ENCODE cell lines [27].

In this chapter, we describe the integrated protocol for poly(A)− RNA separation from both poly(A)+ RNAs and ribosomal RNAs by applying Oligo(dT) separation and RiboMinus depletion. The protocol has been applied in multiple mammalian cell lines which led to the demonstration of non-polyadenylated isoforms of well-characterized lncRNAs and the identification of new classes of lncRNAs.

2 Materials

Solutions are prepared from analytical grade chemicals with deionized DEPC water. Sterilized reagents are aliquoted and stored at room temperature for immediate usage or –20 °C for long-term storage. Ambion® Non-sticky RNase-free 1.5 ml microcentrifuge tubes are from Invitrogen™ (catalog number AM12450). MagneSphere® Technology Magnetic Separation Stand (Magnetic stand, twelve-position) is from Promega (catalog number Z5342).

2.1 Total RNA Purification

1. TRIZOL® Reagent: Invitrogen™, catalog number 15596-018.
2. Chloroform: Sigma-Aldrich®, catalog number 288306.
3. Ambion DNA-*free*™ kit (RNase-free DNase I): Invitrogen™, catalog number AM1906.
4. DEPC-treated water: add diethylpyrocarbonate (DEPC, Sigma-Aldrich®, catalog number 40718) to deionized water to 0.1 % (v/v). Mix thoroughly and let stand at room temperature overnight and autoclave.
5. Isopropyl alcohol: Sigma-Aldrich®, catalog number 278475.
6. 75 % ethanol (v/v): transfer 750 ml absolute ethanol to a 1,000 ml graduated cylinder and add DEPC-treated water to make up to 1 l. Mix well and aliquot.

2.2 Oligo(dT) Selection

1. Dynabeads® Oligo $(dT)_{25}$ beads: Invitrogen™, catalog number 61002.
2. 4 M LiCl solution: weigh 16.956 g LiCl (Sigma-Aldrich®, catalog number L9650-500G) and transfer to a beaker with 50 ml DEPC-treated water. Mix thoroughly and then transfer the solution to 100 ml graduated cylinder. Make up to 100 ml with deionized DEPC water, and filter through a 0.22 μm Millex-GP Syringe Filter Unit (Millipore, catalog number SLGP05010).
3. 1 M Tris–HCl, pH 7.5: weigh 121 g Tris base (Amresco, catalog number 0497-5KG), and transfer to a beaker with 500 ml deionized DEPC water. Mix well and adjust pH to 7.5 with 1 M HCl. Make it up to 1 l with a 1,000 ml graduated cylinder, and filter through a 0.22 μm Millex-GP Syringe Filter Unit.
4. 0.5 M EDTA, pH 7.5: weigh 186.1 g $Na_2EDTA \cdot 2H_2O$ (Sigma-Aldrich®, catalog number E5134-250G), and transfer to a beaker with 500 ml deionized DEPC water. Mix well and adjust pH to 7.5 with 10 M NaOH. Make it up to 1 l with a 1,000 ml graduated cylinder, and filter through a 0.22 μm Millex-GP Syringe Filter Unit.
5. UltraPure™ Glycogen: Invitrogen™, catalog number 10814-010.

6. Binding Buffer: 20 mM Tris–HCl, pH 7.5, 1 M LiCl, 2 mM EDTA.
7. Washing Buffer B: 10 mM Tris–HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA.
8. Elution Buffer: 10 mM Tris–HCl, pH 7.5.
9. Reconditioning Solution: 0.1 M NaOH.
10. Storage Buffer: 250 mM Tris–HCl, pH 7.5, 20 mM EDTA, 0.1 % Tween-20 (Amresco®, catalog number 0777).
11. Absolute ethanol: pre-chilled at –20 °C before use.

2.3 Ribosomal RNA Removal

1. RiboMinus™ Human/Mouse Transcriptome Isolation Kit: Invitrogen™, catalog number K1550-01. All solutions are included.
2. UltraPure™ Glycogen: Invitrogen™, catalog number 10814-010.

2.4 Validation

1. SuperScript® III Reverse Transcriptase: Invitrogen™, catalog number 18080-044.
2. LongAmp™ *Taq* DNA Polymerase: New England BioLabs®, catalog number M0323L.
3. Random Hexamer (6 mer): Takara, catalog number D3801.

3 Methods

3.1 Total RNA Preparation and Quality Control

1. Total RNAs from cultured cells are extracted with Invitrogen TRIZOL® reagent according to the manufacturer's protocol. Briefly, $5–10 \times 10^6$ cells are lysed with 1 ml TRIZOL reagent by passing through a pipette, add 0.2 ml of chloroform per 1 ml of TRIZOL reagent for phase separation, add 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL to precipitate RNAs, centrifuge at $12{,}000 \times g$ for 15 min at 2–8 °C, wash with 75 % ethanol for twice, and redissolve with DEPC-treated water after air dry.
2. RNA quantity and purity is determined by measuring UV absorption with a spectrophotometer. RNA concentration is determined by the OD reading at 260 nm. The value of A_{260}/A_{280} ratio is used as an indication for RNA purity, acceptable from 1.8 to 2.0. RNA integrity is determined by running a standard agarose gel. RNAs are aliquoted into new 1.5 ml non-sticky RNase-free microcentrifuge tubes, about 5–10 μg per tube and stored at –80 °C for long-term storage (*see* **Notes 1** and **2**).
3. An aliquot RNA sample (5–10 μg) is treated with RNase-free DNase I according to the manufacturer's protocol. After treatment, RNAs are separated with chloroform, precipitated

with absolute alcohol, washed with 75 % ethanol and resuspended with 20 μl DEPC-treated water. Place the tube on ice before immediate use or −80 °C for long–term storage (*see* **Notes 1** and **2**).

3.2 Separation of Non-polyadenylated RNAs from total RNAs

3.2.1 Pretreatment of Dynabeads Oligo $(dT)_{25}$

1. Resuspend Dynabeads® Oligo $(dT)_{25}$ beads thoroughly and transfer 200 μl of beads to a 1.5 ml non-sticky RNase-free microcentrifuge tube (*see* **Note 3**). Place the tube on a magnetic stand for 1–2 min. Dynabeads® Oligo $(dT)_{25}$ beads (in brown color) will migrate to the side of the tube nearest to the magnetic stand. Carefully remove the supernatant with pipette while the tube remains on the magnetic stand (*see* **Note 4**).
2. Resuspend beads with 200 μl Binding Buffer and mix gently by pipetting several times (*see* **Note 5**). Put the tube back on the magnetic stand for 1–2 min. Carefully remove the supernatant with the tube on the magnetic stand (*see* **Note 4**). Repeat this wash step twice.
3. Resuspend beads in 200 μl Binding Buffer. Aliquot 100 μl of solution with resuspended beads to two of non-sticky RNase-free microcentrifuge tubes and label as #1 and #2, respectively.

3.2.2 Fractionation of Non-polyadenylated RNA Transcripts from Total RNA Sample

4. Add DEPC-treated water to adjust the volume of 5 μg total RNAs to 50 μl. Denature total RNAs by heating in a 65 °C water bath for 5 min, and then immediately place on ice for 2 min.
5. Place tubes #1 and #2 (**step 3** of Subheading 3.2.1) on magnetic stand for 1–2 min and remove supernatant. Add 50 μl fresh Binding Buffer to resuspend Dynabeads® Oligo $(dT)_{25}$ beads thoroughly, and leave on a rack before use.
6. Add 50 μl denatured total RNA solution into tube #1. Mix well by vortexing gently for 5 min.
7. Place tube #1 back on magnetic stand for 1–2 min, and carefully transfer 100 μl supernatant to tube #2. Mix tube #2 well by vortexing gently at room temperature for 5 min. Meanwhile, keep tube #1 on ice.
8. Put tube #2 on magnetic stand for 1–2 min, and carefully transfer 100 μl supernatant to a new 1.5 ml RNase-free non-sticky microcentrifuge tube. Add 20 μl 4 M LiCl, 1 μl Glycogen, and 250 μl pre-chilled absolute ethanol to precipitate non-polyadenylated RNA transcripts (poly(A)- RNAs). Store at −80 °C until use.
9. Add 100 μl Washing Buffer B to tube #1 (**step 7**), and mix well by pipetting. Place on magnetic stand for 1–2 min and remove supernatant. Repeat this wash step twice. Gently aspirate and discard supernatant.

10. Add 22 µl Elution Buffer to tube #1, and heat at 80 °C for 2 min to elute polyadenylated RNAs from Dynabeads® Oligo $(dT)_{25}$ beads. After heating, put tube #1 immediately back on magnetic stand for 1–2 min, and carefully transfer 20 µl supernatant to a new 1.5 ml RNase-free non-sticky 1.5 ml microcentrifuge tube. Do not disturb the beads (*see* **Note 6**).
11. Add ~80 µl Binding Buffer to 20 µl solution with eluted polyadenylated RNAs. Denature at 65 °C for 5 min in water bath or heat block, and then immediately place the tube on ice for 2 min.
12. Wash the beads in tube #1 with 200 µl Binding Buffer (*see* **Note 5**). Place on Magnetic Stand for 1–2 min and remove supernatant (*see* **Note 4**). Add 100 µl denatured RNA solution from **step 11** into tube #1. Mix well by vortexing gently at RT for 5 min.
13. Place tube #1 back on magnetic stand for 1–2 min, and remove supernatant. Repeat this wash step twice. Gently aspirate and discard supernatant.
14. Add 22 µl Elution Buffer to tube #1, and heat at 80 °C for 2 min to elute polyadenylated RNAs from Dynabeads® Oligo $(dT)_{25}$ beads. After heating, put tube #1 immediately back on magnetic stand for 1–2 min, and carefully transfer 20 µl supernatant to a new 1.5 ml RNase-free non-sticky 1.5 ml microcentrifuge tube. Do not disturb the beads (*see* **Note 6**). Supernatant contains polyadenylated RNA transcripts (poly(A)+ RNAs). Aliquot and store at −80 °C. This is a good stopping point in the process (*see* **Note 1**).

3.2.3 Regeneration of Dynabeads® Oligo $(dT)_{25}$ Beads

15. Dynabeads Oligo $(dT)_{25}$ beads are regenerated according to the manufacturer's protocol. Briefly, beads are washed with 200 µl Reconditioning Solution for three times and with 200 µl Storage Buffer until the pH reaches to 8.0. After magnetic separation, recycled beads are resuspended with Storage Buffer and store at 2–8 °C (*see* **Note 7**).

3.3 Ribosomal RNA Removal

1. Get precipitated poly(A)- RNAs in ethanol from −80 °C (**step 8** of Subheading 3.2), centrifuge at 12,000 × *g* for 15 min at 2–8 °C. Dissolve with 20 µl DEPC-treated water after 75 % ethanol wash and air dry.
2. Resuspend RiboMinus™ Magnetic beads thoroughly and transfer 500 µl of beads to a 1.5 ml non-sticky RNase-free microcentrifuge tube (*see* **Note 3**). Place the tube on a magnetic stand for 1–2 min. RiboMinus™ Magnetic beads (in brown color) will migrate to the side of the tube nearest to the

magnetic stand. Carefully remove the supernatant with pipette while the tube remains on the magnetic stand (*see* **Note 4**).

3. Wash RiboMinus™ Magnetic beads with 500 µl DEPC-treated water twice and discard supernatant after magnetic separation (*see* **Note 5**). Wash with 300 µl Hybridization Buffer once and discard supernatant after magnetic separation (*see* **Note 4**).
4. Resuspend RiboMinus™ Magnetic beads in 400 µl Hybridization Buffer. Aliquot into two RNase-free non-sticky microcentrifuge tubes with 200 µl resuspended beads and label as #A and #B. Incubate beads in a 37 °C water bath before use.
5. Mix 20 µl resuspended non-polyadenylated RNA sample, with 8 µl RiboMinus™ Probe (100 pmol/µl) and 300 µl Hybridization Buffer. Incubate at 70 °C for 5 min to denature RNAs. Allow the sample to slowly cool down to 37 °C over a period of 30 min by placing the tube in a 37 °C water bath.
6. Transfer ~328 µl of denatured hybridized RNA solution to RiboMinus™ Magnetic beads in tube #A (**step 4**), and mix well by pipetting. Incubate the tube at 37 °C for 15 min. During incubation, gently mix the contents occasionally (briefly vortex every 3 min).
7. Place tube #A on magnetic stand for 1–2 min. At the same time, place tube #B (**step 4**) on magnetic stand for 1–2 min and discard supernatant before immediate use. Carefully transfer the supernatant from tube #A to tube #B. Incubate tube #B at 37 °C for 15 min, gently mix the contents occasionally by briefly vortexing every 3 min (*see* **Note 8**).
8. Place tube #B on magnetic stand for 1–2 min, and carefully transfer supernatant to 1.5 ml RNase-free microcentrifuge tubes, 260 µl per tube for two tubes. In each tube, add 50 µl 4 M LiCl, 2 µl Glycogen and 625 µl pre-chilled absolute ethanol to precipitate non-polyadenylated ribosomal-free RNA transcripts (termed as "poly(A)−/ribo− RNAs" thereafter). Store at −80 °C until use. This is a good stopping point in the process (*see* **Note 1**).

3.4 Validation and Applications

1. Here, we applied an integrated method for poly(A)-ribo− RNA preparation by depleting poly(A)+ RNAs and then removing ribosomal RNAs, as indicated in Fig. 1. A similar strategy was also applied for poly(A)− RNA preparation by removing ribosomal RNAs first and then separating poly(A)+ RNAs (*see* **Note 9**).
2. Get precipitation solution with poly(A)−/ribo− RNAs from −80 °C (step 8 of Subheading 3.3), centrifuge at 12,000 × *g* for 15 min at 2–8 °C, wash with 75 % ethanol wash for twice and air dry. Dissolve with 10 µl DEPC-treated water for each tube and combine RNA solution.

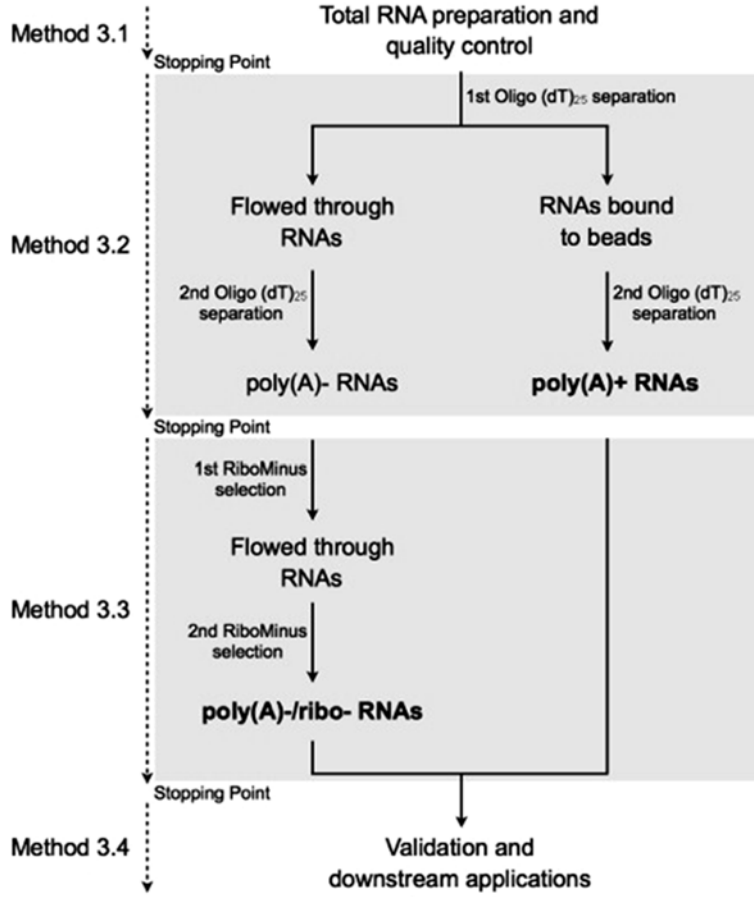

Fig. 1 Diagram of poly(A)−/ribo− RNA preparation. Total RNAs were treated with DNaseI before being subjected to polyadenylated and non-polyadenylated RNA enrichment. See text for details

3. For primary quality control, 1–2 μl of polyadenylated (poly(A)+, **step 14** of Subheading 3.2) and non-polyadenylated (poly(A)−/ribo−, **step 1** of Subheading 3.4) RNA samples are determined by running a standard agarose gel to examine existence of ribosomal RNA (rRNA) bands. Original total RNAs were also used as control. The 28S and 18S ribosomal RNA bands can be detected from total RNAs, but not in samples from poly(A)+ or poly(A)−/ribo− RNA samples (Fig. 2a) [26].
4. For extensive quality control, equal volume of poly(A)+ or poly(A)−/ribo− RNAs from the same preparation are reversed transcribed with random hexamer according to the manufacturer's protocol. PCR amplification is performed according to the manufacturer's protocol with gene-specific primer sets to examine the existence of marker transcripts with (poly(A)+) or

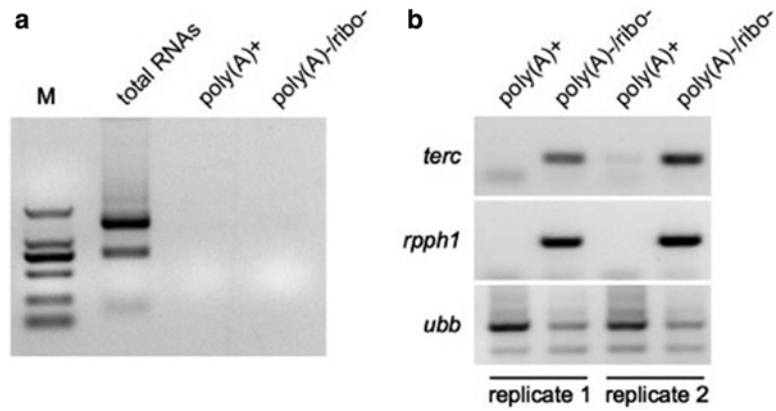

Fig. 2 Validation of poly(A)–/ribo– RNA preparation. (**a**) Agarose gel electrophoresis to confirm the poly(A)–/ribo– RNA preparation. Note that 28S and 18S ribosomal RNA bands cannot be detected in samples from both poly(A)+ (**step 14** of Subheading 3.2) and poly(A)–/ribo– RNA samples (**step 1** of Subheading 3.4). See text for details. (**b**) RT-PCR validation of selected poly(A)–/ribo– transcripts from two replicates. Semi-quantitative RT-PCR was performed with poly(A)–/ribo– sample preparations, and RT-PCR in poly(A)+ sample was used as control. RNAs of *rpph1* and *terc* are selected as marker transcripts without polyadenylated tail. RNA of *ubb* is selected as marker transcript with polyadenylated tail

without (poly(A)–/ribo–) polyadenylated tail (Fig. 2b). Equal amount of PCR products is separated on standard agarose gel. RNAs of *rpph1* (forward primer: CTGAGTGCGTCCTGT CACTC and reverse primer: GAGGAGAGTAGTCTGAATT GG) and *terc* (forward primer: TTTCTCGCTGACTTTCAGC and reverse primer: ACTCGCTCCGTTCCTCTTC) are used as marker transcripts without polyadenylated tail [26]. RNA of *ubb* (forward primer: GTGGACGTGGTTGGTGATTG and reverse primer: GCTCCACCTCCAGAGTGATG) is used as marker transcript with polyadenylated tail (*see* **Note 10**). More maker transcripts and their primer sets for validation can be found in the literature [26].

5. The non-polyadenylated (poly(A)–/ribo–) RNAs can be further applied for many applications, such as RNA-seq. Isolated poly(A)–/ribo– RNA sample can be directly used for library preparation according to the manufacturer's instructions, and then subjected to deep sequencing. RNA-seq reads are mapped to UCSC hg19 human genome annotation with proper aligners (for example, Bowtie [28] or Tophat [29]). For visualization, Bigwig files (.bw) are generated using UCSC bedGraphToBigWig from Bedgraph files, which are generated using genomeCoverageBed_2.13.3 from Tophat generated.bam or Bowtie generated .sam files. The UCSC browser session with uploaded Bigwig (.bw) track files of

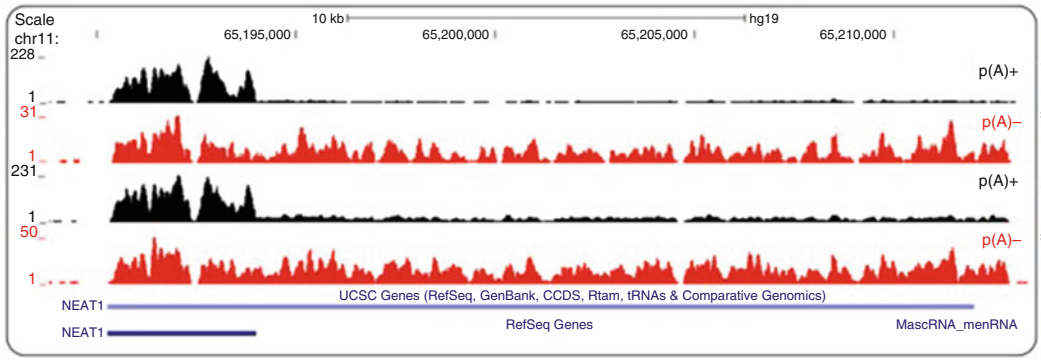

Fig. 3 An example of long noncoding RNA, *NEAT1*, identified with both poly(A)+ (in *black*, p(A)+ for short) and poly(A)−/ribo− (in *red*, p(A)− for short) RNA-seq signals

p(A)+ (in black) and p(A)−/ribo− (in red) reads can be applied for non-polyadenylated and polyadenylated RNA comparison [16, 20]. Long noncoding RNA *NEAT1* was exemplified in this chapter to show its both non-polyadenylated and polyadenylated isoforms. As indicated in Fig. 3, *NEAT1* lncRNA has a short polyadenylated and a long non-polyadenylated isoforms. Note that the short *NEAT1* is expressed in a higher level than the long one does, which is also the case in different cell lines (data not shown).

4 Notes

1. RNA samples/fractionations are recommended to be used immediately for analysis or long-term storage at −80 °C for later usage. The protocol can be safely stopped here.
2. Avoid repeated freeze–thaw of RNA samples. If needed, aliquot RNA samples to small vials before long-term storage at −80 °C.
3. Before usage, resuspend magnetic beads (including Dynabeads® Oligo $(dT)_{25}$ beads in Subheading 3.2 and RiboMinus™ Magnetic beads in Subheading 3.3) and mix well to obtain a homogeneous dispersion in brown color.
4. To separate beads from solution, put the tube back on the magnetic stand for 1–2 min and observe that magnetic beads migrate to the side of the tube nearest the magnetic stand. Carefully aspirate all the supernatants by pipetting with the tube placing on the magnetic stand.
5. To resuspend/wash beads, take tubes from the magnetic stand and mix gently by pipetting several times to obtain a homogeneous dispersion.

6. Add 10 % extra buffer to elute RNAs from beads, and carefully transfer supernatant to a new tube. Do not disturb the magnetic beads.
7. Oligo $(dT)_{25}$ beads can be reused for several times. To avoid any carryover of RNA between different preparations, the beads should be regenerated by following restrict washing and reconditioning. Do not mix regenerated beads with the original fresh stock.
8. For better separation, two cycles of binding can be performed with aliquoted RiboMinus™ Magnetic beads.
9. In this protocol, we purify poly(A)−/ribo− RNAs by depleting poly(A)+ RNAs first and then removing ribosomal RNAs. Other researchers also performed ribosomal RNA removal first and then poly(A)+/poly(A)− separation [27]. We assume that both protocols are sufficient for poly(A)−/ribo− RNA purification. However, since exposed in solution for longer time in the latter protocol, poly(A)+ RNAs may be subjected to partial degradation and hence affect their fractionation from poly(A)− RNAs.
10. Many polyadenylated mRNAs, including *ubb*, show weak, but detectable RT-PCR/RT-qPCR signal in non-polyadenylated fractionation. Although we cannot exclude the degradation and/or incomplete separation during sample preparation, accumulated lines of evidences suggest that lots of RNAs exist in bimorphic transcripts, with both polyadenylated and non-polyadenylated formats, which has been indicated in literatures [10–12, 26].

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