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Detection and Quantitative Analysis of Small RNAs by PCR

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Abstract

Increasing lines of evidence have shown that small non-coding RNAs which were identified in the past several years including miRNAs, piRNAs, rasiRNAs, 21U endo-siRNAs and snoRNAs, appear to be involved in many critical biological processes. Functional studies of these small RNAs require a simple, sensitive and reliable method for detecting and quantifying levels of small RNAs. Here, we describe such a method. This method has been widely used for the validation of cloned small RNAs and also for quantitative analyses of small RNAs in both tissues and cells.

Keywords

small RNAs; miRNAs; piRNAs; expression; PCR

1. Introduction

The past several years have witnessed the surprising discovery of numerous non-coding small RNAs species encoded by genomes of virtually all species [1–6], which include microRNAs (miRNAs)[7–10], piwi-interacting RNAs (piRNAs)[11–14], repeat-associated siRNAs (RasiRNAs)[15–18], 21U endo-siRNAs[19], and small nucleolar RNAs (snoRNAs) [20]. These small RNAs are involved in all aspects of cellular functions through direct or indirect interactions with genomic DNA, RNAs and proteins. Functional studies on these small RNAs are just beginning and some preliminary findings have suggested that they are involved in regulating genome stability, epigenetic marking, transcription, translation, and protein functions [5,21–23]. An easy and sensitive method to detect and quantify levels of these small RNAs in organs or cells during developmental courses, or under different physiological and pathophysiological conditions, is essential for functional studies. Quantitative analyses of small RNAs appear to be challenging because of their small sizes [~20 nucleotides (nt) for miRNAs, ~30 nt for piRNAs and 60–200 nt for snoRNAs]. Northern Blot analysis has been a standard method for detection and quantitative analyses of RNAs. But it requires a relatively large amount of starting material (10–20 µg of total RNA or >5 µg of small RNA fraction). It is also a labor-intensive procedure involving the use of polyacrylamide gel electrophoresis, electro-transfer, radioisotope-labeled probes and autoradiography. We have developed a simple and reliable PCR-based method for detection and quantification of all types of small non-coding RNAs. In this method (Fig. 1), small RNA fractions are isolated and polyA tails are added to the 3' ends by polyadenylation. Small RNA cDNAs (srcDNAs) are then generated by reverse transcription using a primer consisting of adaptor sequences at the 5' end and polyT at the 3' end (miRTQ). Using the srcDNAs, non-quantitative or quantitative PCRs can then be performed using a small RNA-specific primer and the miRTQ primer. This method has been utilized by investigators in

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numerous studies [18,24–38]. Two recent technologies, 454 sequencing and microarray [39,40] for high-throughput analyses of miRNAs and other small RNAs, also need an independent method for validation. 454 sequencing, the next generation sequencing technology, allows virtually exhaustive sequencing of all small RNA species within a small RNA library. However, each of the cloned novel small RNAs need to be validated by examining its expression in organs or cells. Microarray assays of microRNAs have been available although only known or bioinformatically predicted miRNAs are covered. Similar to mRNA microarray analyses, the up- or down-regulation of miRNA levels under different conditions need to be further validated using conventional Northern blot analyses or PCR-based methods like the one that we are describing here.

2. Materials

2.1. Isolation of Small RNAs

mirVana miRNA isolation kit (Ambion), Phosphate buffered saline (PBS) buffer (SIGMA-ALDRICH)

2.2. Polyadenylation

Poly(A) Polymerase (Ambion)

2.3. Purification

mirVana Probe & Marker kit (Ambion)

2.4. Reverse Transcription

Superscript III First-Strand synthesis System for RT-PCR (Invitrogen), miRTQ (see Table 1)

2.5. PCR and qPCR

AmpliTaQ Gold PCR Master Mix (Applied Biosystems) for PCR or SYBR Green PCR Master Mix (Applied Biosystems) for qPCR, a miRNA-specific primer (e.g. let-7a) and miPQ-rev (see Table 1), agarose (Fisher Scientific), 100 bp DNA ladder (Promega)

3. Methods

3.1. Isolation of Small RNAs

1. Harvest tissue (≥ 250 mg) or cells in a 1.7 ml tube with 500 μ l of cold PBS.
2. Centrifuge at $\sim 5,000$ rpm for 2 min at room temperature (RT).
3. Remove PBS as much as possible.
* For cells, remove PBS carefully without breaking the pellet, leave ~ 100 μ l of PBS, and resuspend cells by tapping gently.
4. Add 300–600 μ l of Lysis/Binding buffer (10 volumes per tissue mass) on ice.
* When you start with frozen tissue or cells, immediately add Lysis/Binding buffer (10 volumes per tissue mass) on ice.
5. Cut tissue into small pieces using scissors, and grind it using a homogenizer
* For cells, skip this step.
6. Vortex for 40 sec to mix.
7. Add 1/10 volume of miRNA Homogenate Additive on ice, mix well by vortexing.

8. Leave the mixture on ice for 10 min.
 - * For tissue, mix it every 2 min.
9. Add an equal volume (330–660 μ l) of Acid-Phenol: Chloroform
 - * Be sure to withdraw from the bottom phase (the upper phase is an aqueous buffer).
10. Mix thoroughly by inverting the tubes several times.
11. Centrifuge at 10,000 rpm for 5 min at RT.
12. Recover the aqueous phase carefully without disrupting the lower phase, and transfer it to a fresh tube.
13. Measure the volume using a scale (1g = ~1 ml) and note it.
14. Add 1/3 volume of 100% ethanol at RT to the recovered aqueous phase.
15. Mix thoroughly by inverting the tubes several times.
16. Transfer up to 700 μ l of the mixture into a Filter Cartridge within a Collection Tube.
 - * Label the filter as 'total RNA'
 - * When you have >700 μ l of the mixture, apply it in successive application to the same filter.
17. Centrifuge at 10,000 rpm for 15 sec at RT.
18. Collect the filtrate (the flow-through).
 - * Save the Cartridge for total RNA isolation (go to #24)
19. Add 2/3 volume of 100% ethanol at RT to the flow-through.
20. Mix thoroughly by inverting the tubes several times.
21. Transfer up to 700 μ l of the mixture into a new Filter Cartridge.
 - * Label the filter as 'small RNA'
 - * When you have >700 μ l of the filtrate mixture, apply it in successive application to the same filter.
22. Centrifuge at 10,000 rpm for 15 sec at RT.
23. Discard the flow-through, and repeat until all of the filtrate mixture is through the filter. Reuse the collection tube for the following washing steps.
24. Apply 700 μ l of miRNA Wash Solution 1 (working solution mixed with ethanol) to the filter.
25. Centrifuge at 10,000 rpm for 15 sec at RT.
26. Discard the flow-through.
27. Apply 500 μ l of miRNA Wash Solution 2/3 (working solution mixed with ethanol) to the filter.
28. Centrifuge at 10,000 rpm for 15 sec at RT.
29. Discard the flow-through and repeat #27.
30. Centrifuge at 12,000 rpm for 1 min at RT.

31. Transfer the filter to a new Collection Tube
32. Apply 100 μ l of pre-heated (95 °C) Elution Solution or RNase-free water to the center of the filter, and close the cap.
* Aliquot a desired amount of Elution Solution into a 1.7 ml tube, heat it on a heat block at 95 °C for ~15 min. Open the cap carefully because it might be splash due to pressure build up.
33. Leave the filter tube for 1 min at RT.
34. Centrifuge at 12,000 rpm for 1 min at RT.
35. Measure total RNA and small RNA concentration using NanoDrop or another spectrophotometer.
36. Store it at -80 °C until used.

3.2. Polyadenylation

1. Set up a reaction mixture with a total volume of 50 μ l in a 0.5 ml tube containing 100 ng-2 μ g of small RNAs, 10 μ l of 5 $\times$ E-PAP Buffer, 5 μ l of 25 mM MnCl₂, 5 μ l of 10 mM ATP, 1 μ l (2 U) of *E. coli* Poly(A) Polymerase I and RNase-free water (up to 50 μ l).
* When you have a low concentration of small RNAs, increase the total volume. 5 $\times$ E-PAP Buffer, 25 mM MnCl₂, and 10 mM ATP should be increased accordingly.
2. Mix well and spin the tube briefly.
3. Incubate for 1 hr at 37 °C.

3.3. Purification

1. Add an equal volume (50 μ l) of Acid-Phenol: Chloroform to the polyadenylation reaction mixture.
* When you have ≥ 50 μ l of the mixture, increase Acid-Phenol: Chloroform accordingly.
2. Mix thoroughly by tapping the tube.
3. Centrifuge at 10,000 rpm for 5 min at RT.
4. Recover the aqueous phase carefully without disrupting the lower phase, and transfer it to a fresh tube.
5. Add 12 volumes (600 μ l) of Binding/Washing buffer to the aqueous phase.
* When you have ≥ 50 μ l of the aqueous phase, increase Binding/Washing buffer accordingly.
6. Transfer up to 460 μ l of the mixture into a Purification Cartridge within a Collection Tube.
7. Centrifuge at 10,000 rpm for 15 sec at RT.
8. Discard the filtrate (the flow-through), and repeat until all of the mixture is through the cartridge. Reuse the collection tube.
9. Apply 300 μ l of Binding/Washing buffer to the cartridge.
10. Centrifuge at 12,000 rpm for 1 min at RT.

11. Transfer the cartridge to a new collection tube.
12. Apply 25 μ l of pre-heated (95 °C) Elution Solution to the center of the filter, and close the cap.
* Aliquot a desired amount of Elution Solution into a 1.7 ml tube, heat it on a heat block at 95 °C for ~15 min. Open the cap carefully because it might be splash due to pressure build up.
13. Let the filter tube stand for 1 min at RT.
14. Centrifuge at 12,000 rpm for 1 min at RT.
15. Repeat #12–14 with a second aliquot of 25 μ l of pre-heated (95 °C) Elution Solution.
16. Measure tailed RNA concentration using NanoDrop or another spectrophotometer.
17. Store it at –80 °C until used.
* After polyadenylation, RNA concentration should increase up to 5–10 times of the starting concentration.

3.4. Reverse Transcription

1. Mix 2 μ g of tailed RNAs, 1 μ l (1 μ g) of miRTQ, and RNase-free water (up to 21 μ l) in a PCR tube.
2. Incubate for 10 min at 65 °C and for 5 min at 4 °C.
3. Add 1 μ l of 10 mM dNTP mix, 1 μ l of RNaseOUT, 4 μ l of 10 $\times$ RT buffer, 4 μ l of 0.1 M DTT, 8 μ l of 25 mM MgCl₂, and 1 μ l of SuperScript III Reverse Transcriptase the mixture.
* When you have a low concentration of ligated RNAs, increase the total volume. 10 $\times$ RT buffer, 0.1 M DTT, and 25 mM MgCl₂ should be increased accordingly.
4. Mix well and spin the tube briefly.
5. Incubate for 60 min at 50 °C and for 5 min at 85 °C for inactivation of the reaction.
6. Add 1 μ l of RNase H to the mixture.
7. Incubate for 20 min at 37 °C.
8. Add 60 μ l of nuclease-free water.

3.5. PCR and qPCR

1. Set a reaction with a total volume of 25 μ l in a PCR tube containing 1 μ l of small RNA cDNAs(srcDNAs), 1 μ l (5 pm) of a miRNA-specific primer (srSP), 1 μ l (5 pm) of RTQ-UNIr, 12.5 μ l of AmpliTaq Gold PCR Master Mix and 9.5 μ l of nuclease-free water.
* For qPCR, use SYBR Green PCR Master Mix instead of AmpliTaq Gold PCR Master Mix.
2. Mix well and spin the tube briefly.
3. Start PCR or qPCR with the conditions: [at 95°C for 10 min, then 40 cycles (at 95°C for 15 sec, and at 60°C for 1 min)].
4. Run 2 μ l of the PCR or qPCR products along with a 100 bp DNA ladder on a 2% agarose gel.

* PCR products should be ~120–200 bp depending on small RNA species (e.g. ~120–130 bp for miRNAs and piRNAs).

4. Notes

1. This PCR method can be used for quantitative PCR (qPCR) or semi-quantitative PCR (semi-qPCR) on small RNAs such as miRNAs, piRNAs, snoRNAs, small interfering RNAs (siRNAs), transfer RNAs, (tRNAs) and ribosomal RNAs (rRNAs) [18,24–38].
2. Design miRNA-specific primers to contain only the “core sequence” since our cloning method uses two degenerate nucleotides (VN) at the 3' end to make small RNA cDNAs (srcDNAs) (see let-7a in Table 1).
3. For qPCR analysis, two miRNAs and a piRNA were quantitated using the SYBR green Green PCR Master Mix (see ref. 41). Cycle threshold (Ct) is the cycle number at which the fluorescence signal reaches the threshold level above background. A Ct value for each miRNA tested was automatically calculated by setting a threshold level to be 0.1–0.3 with auto baseline. All Ct values depend on abundance of target miRNAs. For example, average Ct values for let-7 isoforms range from 17 to 20 when 25 ng of each srcDNA sample from the multiple tissues was used (see ref. 41).
4. This method amplifies over a broad dynamic range up to 10 orders of magnitude and has excellent sensitivity capable of detecting as little as 0.001 ng of the srcDNA in qPCR assays
5. For qPCR, each small RNA-specific primer should be tested along with a known control primer (e.g. let-7a) for PCR efficiency. Good efficiencies range from 90% to 110% calculated from slopes between –3.1 and –3.6.
6. On an agarose gel, mature miRNAs and precursor miRNAs (pre-miRNAs) can be differentiated by their size. PCR products containing miRNAs will be ~120 bp long in size while products containing pre-miRNAs will be ~170 bp long. However, our PCR method preferentially amplifies mature miRNAs [see Results and Discussion in ref. 41]. We tested our PCR method to quantify over hundred miRNAs, but never detected pre-miRNAs [18,29–31,38].

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Fig. 1.

Overview of small RNA complementary DNA (srcDNA) library construction for PCR or qPCR analysis. Small RNAs are polyadenylated using a polyA polymerase. The polyA-tailed RNAs are reverse-transcribed using a primer miRTQ containing oligo dTs flanked by an adaptor sequence. RNAs are removed by RNase H from the srcDNA. The srcDNA is ready for PCR or qPCR to be carried out using a small RNA-specific primer (srSP) and a universal reverse primer, RTQ-UNIr.

Table 1

Oligonucleotides used

Name	Sequence (5' to 3')	Note	Usage
miRTQ	CGAATTCTAGAGCTCGAGGCAGGCGACATGGCT GGCTAGTTAAGCTTGGTACCGAGCTCGGATCCA CTAGTCCTTTTTTTTTTTTTTTTTTTTTTTTTVN	RNase free, HPLC	Reverse transcription
RTQ-UNlr	CGAATTCTAGAGCTCGAGGCAGG	Regular desalting	PCR/qPCR
let-7a	TGAGGTAGTAGGTTGTATAG	Regular desalting	PCR/qPCR

* V = A, C, or G; N = A, C, G, or T