Chapter 11

Nonradioactive Northern Blot of circRNAs

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Abstract

Circular RNAs (circRNAs) are recognized as a special species of transcripts in metazoans with increasing studies, and northern blotting is a direct way to confirm the existence and to evaluate the size of individual circRNAs. Northern blotting probes can be radioactive isotope (^{32}P) labeled, which is not environment-friendly and sometimes inconvenient to use. Here, we describe a nonradioactive northern blot protocol with digoxigenin-labeled probe to detect circRNA.

Key words Nonradioactive, Northern blot, circRNA, Digoxigenin

1 Introduction

Covalently closed circular RNAs (circRNAs) were firstly discovered in plant viroids and later hepatitis delta virus in 1970s [1, 2]. Owning to the advance of high-throughput RNA sequencing and bioinformatics, circRNAs have been gradually recognized as a special class of RNAs in eukaryotic cells [3–7]. When studying circRNA, it is important to confirm experimentally the existence of individual circRNAs. Northern blotting is almost the most convincing approach to examine the existence, abundance, and size of circRNA.

A general northern blotting procedure [8] starts with denatured RNA samples separated by gel electrophoresis. Then the RNAs separated by size are transferred to nylon membrane through capillary or electrical means. Once transfer is finished, RNAs are immobilized through covalent linkage to the membrane by UV light or baking. The labeled probe against specific RNA sequences is then hybridized with the membrane. After washing the membrane, the hybrid signals are then detected with various methods depending on the way of probe labeling.

Classically probes of northern blotting were labeled with radioactive isotopes, mostly ^{32}P [4, 6]. Since radioactive materials are potentially harmful to health and environment, and relatively hard to handle or recycle [9], nonradioactive-labeled probes are

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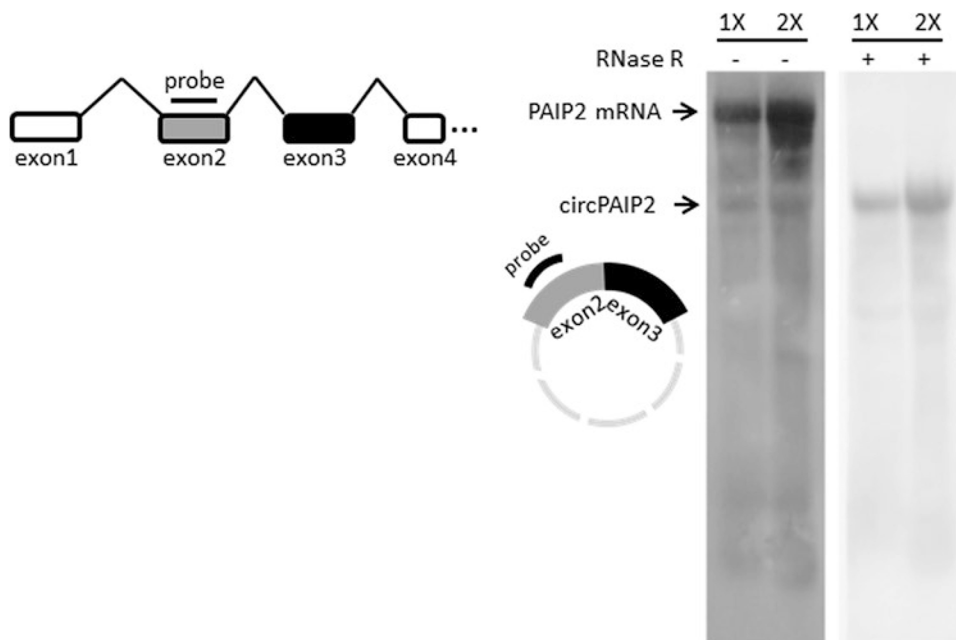

Fig. 1 Image of nonradioactive northern blot of circPAIP2. Sequence composition of PAIP2 mRNA and circRNA with position of the probe are indicated

thus good alternatives [10]. Here, we describe the nonradioactive northern blot for circRNAs with digoxigenin-labeled probe. One probe is designed to detect the circPAIP2 in this particular experiment (Fig. 1) [10]. RNase R is a RNA exonuclease that can degrade linear RNA and keep the circRNA intact, and it is now commonly used to verify the RNA as covalently closed molecule.

2 Materials

Prepare all solutions with RNase-free water, which is purified deionized water pretreated by 0.1% DEPC with autoclave. All compounds used are analytical grade. Filter all solutions through a 0.22 μm Corning filter before use. Prepare and store all reagents at room temperature (unless otherwise specified). Diligently follow all waste disposal requirements when disposing waste materials.

1. 10× MOPS buffer: 4 mol/L 3-(4-Morpholino) propanesulfonic acid (MOPS), 1 mol/L NaOAc, 100 mol/L EDTA, pH 7.0. Weigh 92.4 g MOPS, 13.6 g NaOAc, 7.6 g EDTA, and transfer them to the beaker (*see* **Note 1**). Add RNase-free water to a volume of 900 mL. Mix and adjust pH with NaOH (Add around 8 g) (*see* **Note 2**). Make up to 1 L, filter, and autoclave. Store the buffer in brown bottle to avoid decomposing at 4 °C (*see* **Note 3**).

2. Agarose.
3. Gelred (nucleic acid dye).
4. 37% formaldehyde (*see* **Note 4**).
5. 2× loading buffer.
6. 10× TBE buffer: 0.9 mol/L Tris-borieacid, 0.02 mol/L EDTA, pH 8.3. Weigh 108 g Tris base, 9.2 g EDTA, 55.2 g borieacid, and transfer them to the beaker. Add RNase-free water to a volume of 900 mL. Mix and adjust pH with HCl. Make up to 1 L, filter and autoclave. Store the buffer at room temperature (*see* **Note 5**).
7. Nylon membranes.
8. Labeling mix.
9. Transcription buffer.
10. T7 RNA polymerase.
11. DNase I enzyme.
    8–11 (*see* **Note 6**) were included in Roche DIG RNA Labeling Kit.
12. 20× SSC buffer: 3 mol/L NaCl, 0.3 mol/L sodium citrate, pH 7.0. Weigh 175.3 g NaCl, 88.2 g sodium citrate, and transfer them to the beaker. Add RNase-free water to a volume of 900 mL. Mix and adjust pH with NaOH. Make up to 1 L and filter. Store the buffer at room temperature.
13. 10% SDS: Weigh 10 g SDS and transfer them to the beaker. Add RNase-free water to a volume of 100 mL. Mix, filter, and store the buffer at room temperature (*see* **Note 7**).
14. Washing buffer I: 2× SSC, 0.1%SDS.
15. Washing buffer II: 0.5× SSC, 0.1%SDS.
16. Maleic acid buffer: 0.1 mol/L Maleic acid, 0.15 mol/L NaCl, pH 7.5. Weigh 11.6 g maleic acid, 8.8 g NaCl, and transfer them to the beaker. Add RNase-free water to a volume of 900 mL. Mix and adjust pH with NaOH. Make up to 1 L and filter. Store the buffer at room temperature.
17. Washing buffer: 100 mL Maleic acid buffer add 300 μL Tween 20 (*see* **Note 8**).
18. Detection buffer: 0.1 mol/L Tris–HCl, 0.1 mol/L NaCl, pH 9.5. Weigh 12.1 g Tris base, 5.8 g NaCl, and transfer them to the beaker. Add RNase-free water to a volume of 900 mL. Mix and adjust pH with NaOH. Make up to 1 L and filter. Store the buffer at room temperature.
19. Anti-digoxigenin-AP, Fab fragments (*see* **Note 9**).
20. CDP-Star (the Chemiluminescent Substrate), ready-to-use.
21. DIG Easy Hyb Granules (*see* **Note 10**).
22. Blocking solution.
    19–22 were included in DIG Northern Starter Kit.

3 Methods

Carry out all the procedures at room temperature unless specified.

3.1 1% Formaldehyde Agarose Gel

The concentration of the gel for the length of RNA <1000 nt is 1.5% and ≥ 1000 nt is 1%.

1. Prepare an 1% agarose gel (50 mL) by mixing 0.5 g agarose with 43.5 mL RNase-free water.
2. Microwave the mixture into solution and cool to 55 °C in water bath.
3. Add 5 μL Gelred (10,000×), 5 mL 10× MOPS, and 1.5 mL 37% formaldehyde for the final concentrations of 1×, 1×, and 1%, respectively (*see* **Note 11**).
4. Mix and pour the gel in the hood to avoid toxic formaldehyde fume.
5. Let the gel sit for at least half an hour in hood before use.

3.2 Electrophoresis and Membrane Transfer of RNA

1. Mix RNA samples (10–30 μg total RNA per lane) and 2× loading buffer in tubes.
2. Heat samples at 80 °C for 10 min to denature RNA (*see* **Note 12**), and then place in ice water immediately.
3. Spin samples briefly and load on the gel (*see* **Note 13**).
4. Run the gel at 150 V for 1 h with recirculating buffer (1× MOPS) and take photos (*see* **Note 14**).
5. Soak the gel in a large volume of RNase-free water with gentle shaking to remove the formaldehyde and carefully pre-wet the membrane in RNase-free water for 5 min.
6. Transfer the RNA from the gel to a membrane with wet transfer using 0.5× TBE buffer at 200 mA overnight (*see* **Note 15**).

3.3 Nonradioactive Probe Preparation

1. Design the probe. For circRNA, two kinds of probes can be selected. One is against the continuous exon region. Another kind is against the exon-exon junction of circRNA. Length of the probe is generally 100–200 nt.
2. Prepare the template. The T7 promoter sequences (*see* **Note 16**) are added to PCR primers, and thus are incorporated into the PCR products.
3. Synthesize the digoxigenin-labeled (DIG-labeled) RNA probe by mixing together the following components (Roche DIG RNA Labeling Kit).
   (a) 1 μg DNA template (*see* **Note 17**).
   (b) 4 μL Labeling mix.

(c) 4 μL Transcription buffer.
(d) 2 μL T7 RNA polymerase.
(e) Add RNase-free water to 20 μL.

4. Incubate the mixture at 37 °C for 4 h instead of overnight, then with 1 μL DNase I incubation for 15 min to remove the DNA template.
5. Purify the probe with trizol extraction (Life Technologies) and confirm with RNA gel.

3.4 Hybridization

1. Crosslink RNA to nylon membrane which prefers wet membrane with 2× SSC buffer using UV and dry at 80 °C for 1 h in a hybridization oven.
2. Pre-hybridize with pre-heated DIG Easy Hyb buffer for 2 h with gentle agitation in an appropriate container (*see* **Note 18**).
3. Denature 100 pmol DIG-labeled RNA probe by boiling for 10 min and rapidly cooling in ice water.
4. Mix the denatured DIG-labeled RNA probe with pre-heated DIG Easy Hyb buffer (avoid foaming).
5. Hybridize at 45–68 °C overnight with gentle agitation in a hybridization oven.

3.5 Detection

All the following procedures are according to the protocol of DIG Northern Starter Kit with some modifications.

1. Wash the membrane for 5 min in ample Washing buffer I at 15–25 °C under constant agitation, twice.
2. Wash the membrane for 15 min in ample Washing buffer II at 45–68 °C under constant agitation, twice. These four washes are stringency washes.
3. Rinse the membrane briefly for 5 min in Washing buffer.
4. Incubate the membrane with 10 mL Blocking solution for 1 h.
5. Incubate the membrane with 10 mL Antibody solution (1 μL Anti-digoxigenin-AP antibody diluted in 10 mL Blocking solution) for 1 h.
6. Wash the membrane for 15 min in Washing buffer, twice and equilibrate the membrane in 20 mL Detection buffer for 5 min.
7. Cover the membrane with ample CSPD ready-to-use immediately, and incubate for 10–30 min at 37 °C to enhance the luminescent reaction (*see* **Note 19**).
8. Expose the membrane and take images with an appropriate imager (e.g., ImageQuant LAS4000 Biomolecular Imager, expose for 5–20 min for detection).

4 Notes

1. Adding some water into the beaker first and with magnetic stir bar working help to dissolve the MOPS.
2. When adjusting pH, high concentrated HCl or NaOH can be used firstly to shorten the gap from the starting pH to the required pH. Then the HCl or NaOH of lower ionic strengths could be used until up to the required pH.
3. MOPS buffer is susceptible to bacterial contamination and light decomposition. It is not suitable for experimental use when the color is light yellow.
4. When dealing with formaldehyde, all handling must be in fume hood.
5. 10× TBE is very easy to precipitate, even when it is stored at the room temperature. Stir with magnetic bar in a 37 °C water bath to fully dissolve the solution before use.
6. All the components must be stored at −20 °C and avoid alternate freezing.
7. Do not place 10% SDS on ice, because of its rapid and easy precipitation at low temperature.
8. Tween 20 is very viscous and susceptible to sticking on the pipette; using pipettes with tips cut to aliquot Tween 20 is essential.
9. Centrifuge before use.
10. Add 64 mL RNase-free water into DIG Easy Hyb granule immediately. DIG Easy Hyb solution is stable at the room temperature for 1 month.
11. Gelred, 10× MOPS, and 37% formaldehyde can mix together and pre-warm in a 55 °C water bath. And then add the mixture along the wall of the glass beaker to avoid solidification and foaming.
12. Denature the samples at 100 °C for 5 min, which can disrupt the RNA secondary structure.
13. The suggested volume is no more than 60 μL, and the maximum amount of total RNA is 50 μg per lane.
14. Run the gel briefly for 10–15 min; taking photos to visualize ribosomal RNA bands helps to show equal loading of total RNAs.
15. Use fresh diluted 0.5× TBE transfer buffer. Wet transfer needs to be carried out in ice water bath.
16. When designing the primer, make sure the T7 promoter sequence TAATACGACTCACTATAGGG was added, so that this sequence is in 5′ of the antisense strand to the interested

RNA. The first 17 nt is the core region of T7 promoter. The three Gs followed can enhance the transcriptional efficiency.

17. The templates need to be purified with Gel cycle and dry in air for longer time (~20 min) to remove moisture completely, and this can enhance the transcriptional efficiency.
18. UVP molecular hybrid tube is recommended for pre-hybridization. And bubbles between the membrane and the wall of hybrid tube need to be avoided to decrease background.
19. Appropriate increase of the incubation time in “CSPD ready-to-use” can sometimes get clearer image.

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References

1. Sanger HL, Klotz G, Riesner D et al (1976) Viroids are single-stranded covalently closed circular RNA molecules existing as highly base-paired rod-like structures. Proc Natl Acad Sci U S A 73(11):3852–3856
2. Kos A, Dijkema R, Arnberg AC, van der Meide PH, Schellekens H (1986) The hepatitis delta (delta) virus possesses a circular RNA. Nature 323:558–560
3. Dixon RJ, Eperon IC, Hall L, Samani NJ (2005) A genome-wide survey demonstrates widespread non-linear mRNA in expressed sequences from multiple species. Nucleic Acids Res 33:5904–5913
4. Hansen TB et al (2013) Natural RNA circles function as efficient microRNA sponges. Nature 495:384–388
5. Jeck WR et al (2013) Circular RNAs are abundant, conserved, and associated with ALU repeats. RNA 19:141–157
6. Memczak S et al (2013) Circular RNAs are a large class of animal RNAs with regulatory potency. Nature 495:333–338
7. Salzman J, Chen RE, Olsen MN, Wang PL, Brown PO (2013) Cell-type specific features of circular RNA expression. PLoS Genet 9: e1003777
8. Trayhurn P (1996) Northern blotting. Pro Nutr Soc 55:583–589
9. Streit S, Michalski CW, Erkan M, Kleeff J, Friess H (2009) Northern blot analysis for detection and quantification of RNA in pancreatic cancer cells and tissues. Nat Protoc 4(1):37–43
10. Li Z et al (2015) Exon-intron circular RNAs regulate transcription in the nucleus. Nat Struct Mol Biol 22:2959