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Detection of microRNAs by Northern blot analyses using LNA probes

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Abstract

MicroRNAs (miRNAs) are small, endogenous, non-coding RNA species, about 21 nucleotides in length, which modulate the expression of animal and plant target genes at the post-transcriptional level. It has been shown that miRNA based gene regulation plays a pivotal role in pathways involved in growth and development. Understanding miRNA mediated processes requires new technologies enabling efficient detection of small RNA species. Here we report the optimization of a miRNA Northern blot detection method based on LNA (locked nucleic acid)-modified oligonucleotide hybridization. This technique allows sensitive and highly specific detection of mature miRNAs.

Keywords: MicroRNA; LNA (locked nucleic acid); Northern blot

1. Introduction

Northern blot analysis of small RNA species, such as miRNAs, is a widely used technique to assess accumulation levels of miRNAs of interest. This technique, combined with polyacrylamide gel electrophoresis, allows examination of expression properties of target miRNAs, determination of their sizes, and validation of predicted miRNAs. However, the main drawback of this method is its low sensitivity which creates a requirement for large amounts of total RNA for samples. This disadvantage is especially pronounced when monitoring expression of low-abundant miRNAs or when cell or tissue source of RNA samples is limited.

To evade the sensitivity problem of miRNA Northern blotting technology, locked nucleic acid (LNA)-modified oligonucleotide probes were used to enhance the efficiency of hybridization [1]. LNA-modified oligonucleotides have been shown to exhibit improved thermal stability when hybridized to their target molecules [2,3]. LNA monomers are bicyclic, high-affinity RNA analogues having a modified ribose moiety [4]. In LNAs the furanose ring in the sugar–phosphate backbone is chemically locked in an N-type (C3′-endo) conformation by the introduction of a 2′-*O*,4′-*C* methylene bridge. This chemical modification induces a conformational change that brings about enhanced base stacking and phosphate backbone pre-organization [5]. Since LNAs possess similar water solubility as DNAs or RNAs, LNA-modified oligonucleotides can be used easily in many biological experimental applications. Moreover, the introduction of LNA residues results in increased stability against endo- and 3′-exonucleases [6]. The superior properties of LNA-modified oligonucleotides make them an ideal tool for detecting small RNA molecules. In Northern blot hybridization assays, LNA-modified oligonucleotide probes detected mature miRNAs by at least a 10-fold higher efficiency than traditional DNA probes [1]. Moreover, LNA-modified oligonucleotide probes were highly specific, as demonstrated by the use of different single- and double-mismatched LNA probe molecules. The enhanced efficiency, stability and the discriminatory power of LNA-modified oligonucleotide probes render them especially useful in miRNA Northern blotting. LNA-modified oligonucleotide probes were also used for in situ hybridization experiments, indicating their potential in miRNA detection at the tissue level as well

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[7,8]. Here, we describe detailed methods for detecting miRNAs by LNA-modified oligonucleotides in Northern blotting.

2. Method

2.1. Reagents

- TRIZOL (Invitrogen) or TRI Reagent (Sigma).
- 10× TBE buffer: 0.9 M Tris, 0.9 M boric acid, 0.02 M EDTA, pH 8.0.
- Penguin electrophoresis system or equivalent.
- 12% acrylamide solution (can be adjusted between 8 and 15%): 24 mL 40% acrylamide/bis (19/1) solution (Ambion), 8 mL 10× TBE, 40 g urea, and water to 80 mL.
- TEMED (Sigma).
- Ammonium persulfate (APS): Prepare 10% solution in water (immediately freeze in aliquots for single use at −20°C).
- Small RNA loading dye: 10 mL deionized formamide, 200 μL 0.5 M EDTA (pH 8.0), add xylene cyanol and bromphenol blue to obtain a faint blue solution. Avoid a high concentration of dye since it may interfere with the correct separation of small RNA species.
- FDE: 10 mL deionised formamide, 200 μL 0.5 M EDTA (pH 8.0), 10 mg xylene cyanol, 10 mg bromphenol blue.
- Ethidium bromide solution: 10 mg/mL in water.
- Nytran N membrane (Schleicher & Schuell, Germany) or Hybond-N+ membrane (Amersham Pharmacia Biotech).
- 20× SSC: 3 M NaCl, 0.3 M sodium citrate-$2H_2O$, pH 7.0.
- Synthetic RNAs for RNA size markers.
- LNA-modified oligonucleotides for probes (Exiqon, Denmark).
- T4 polynucleotide kinase (Fermentas, Lithuania).
- [γ-^{32}P]ATP or equivalent.
- Hybridization solution: 50% deionized formamide, 0.5% SDS, 5× SSPE, 5× Denhardt's solution (50× stock from Invitrogen), and 20 μg/ml sheared, denatured salmon sperm DNA.
- RNase A stock solution 10 mg/mL.
- RNase A buffer: 0.5 M NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 20 μg/ml RNase A.

2.2. RNA extraction

The extraction of intact, high quality RNA is a prerequisite for the reliable detection of miRNAs. TRIZOL (Invitrogen) and TRI Reagent (Sigma) are well suited for purifying RNA samples of appropriate quality for small RNA detection. Usage of these reagents ensures that the samples will be practically free of genomic DNA and satisfactory good quality. Do not use minicolumn-based purification methods for RNA extraction since these may result in loss of the small RNA fraction. All steps of RNA extraction should be performed according to the manufacturer's instructions. It is important to assess the quality of the sample by analysing 1–5 μL of the extracted RNA sample by electrophoresis in 1× TBE buffer using 1.2% agarose gels containing nuclease-free ethidium bromide (0.5–1 μg/ml final concentration).

2.3. Denaturing PAGE

It is important to ensure proper running conditions during denaturing PAGE since improperly run denaturing gels may result in the appearance of artificial signals. Several types of equipment are available on the market for PAGE. It is important to follow the manufacturers' instructions irrespective of the type of equipment used. We use the Gel Penguin Electrophoresis System, a 20 cm × 20 cm glass plate gel sandwich with 1.5 mm spacers. Make sure that the apparatus is cleaned with detergent and rinsed thoroughly with autoclaved, sterile distilled water. After setting up the gel apparatus using 1.5 mm spacers, prepare 80 mL acrylamide solution by adjusting acrylamide content (about 8–15%) of the gel depending on the required resolution. It is also possible to use thinner spacers; however, in this case, modification of the running conditions is necessary. Warm up the solution carefully by using a microwave oven or water bath to dissolve the urea. Cool the solution to room temperature and bring up to 80 mL with water. Add 480 μL 10% APS and 32 μL TEMED and pour the gel, allowing it to polymerize at least for 1 h. Remove the comb very carefully to maintain intact wells, and assemble the gel sandwich on the apparatus. Rinse wells thoroughly with 1× TBE using a syringe and a needle. No leaks should be observed. The gel must be pre-run at 400 V (40 mA) for 60 min. Make sure that during the pre-run the gel warms up because this guarantees proper denaturing conditions. Before loading the samples on the warm gel, rinse the wells again.

2.4. Sample preparation, gel electrophoresis and capillary gel transfer

Since the advantage of LNA modified oligonucleotide probes is their enhanced sensitivity, the amount of RNA samples used should be adjusted accordingly. Quantities ranging from 1 to 100 μg total RNA can be loaded into the wells. This might be determined by the expected abundance of the small RNA species of interest. Generally, further purification of the small RNA fraction is not necessary. However, if the target miRNA is poorly expressed, the small RNA fraction can be separated from higher molecular weight RNA species and concentrated. The most reliable way to achieve small RNA purification is to purchase a small RNA purification system (for example, the flashPAGE™ Fractionator (Ambion)). Add the adjusted amount of RNA sample in 20 μL water to 20 μL small RNA loading dye. It is possible to reduce the volume

of the samples to about 10 μL (5 μL RNA sample and 5 μL small RNA loading dye) depending on the amount of samples and size of the wells. The reduced sample volume may result in sharper bands. Denature samples at 65 °C for 20 min, chill them on ice for 1 min, and spin in a microcentrifuge for 15 s at high speed. Load samples and markers (see marker preparation in Section 2.5) into wells of the warm gel. Also, 10 μL FDE into each well not used for samples; FDE contains a higher concentration of dye which helps to monitor progress of the electrophoresis.

Run the gel at 200 V while the entire sample volumes enter into the gel, and then run at 400 V until the bromophenol blue reaches the bottom of the gel. Dismantle the apparatus and submerge the gel in 500 mL 1× TBE containing 0.5 μg/ml ethidium bromide to stain tRNAs and 5S RNA for loading control. Apply gentle shaking for 10 min and rinse the gel in 1× TBE for 5–10 min to wash away excess ethidium bromide. Perform the washing step in 1× TBE even if quantification by ethidium bromide staining is not required. An alternative method to check the uniformity of loading is stripping the membrane (see Section 2.8) and re-hybridizing with probes detecting, for example, 5S rRNA.

Transfer of RNAs onto the membrane is performed by capillary blotting subsequently to soaking the gel in 20× SSC for 10 min following routine protocols. Alternatively, the transfer can be performed using a semi-dry transfer cell (e.g., Bio-Rad) according the manufacturer's instructions. After blotting, rinse the membrane with 2× SSC for 2 min and allow it to dry. Using a pencil, mark the positions of lanes on the RNA sample side of the membrane. Fix RNA onto the membrane by ultraviolet cross-linking (with the RNA sample side facing up); for example, use the Stratalinker® UV Crosslinker (Stratagene) according to the manufacturer's instructions.

2.5. Preparation of radiolabelled, LNA-modified oligonucleotide probes for miRNA detection

LNA-modified oligonucleotide (Exiqon, Denmark) complementary to the target miRNA is labelled by combining 10 pmol of LNA-modified oligonucleotide with 1 μL 10× T4 polynucleotide kinase buffer and then bringing the reaction volume to 8.5 μL with water. (Exiqon has a website for probe design; see http://lnatools.com.) Then, 0.5 μL T4 polynucleotide kinase and 1 μL [γ-^{32}P]ATP (0.4 MBq) are added in this order, and the reaction is incubated at 37 °C for 60 min. Forty milliliters of 1× TE is added to the reaction and the efficiency of labelling can then be estimated by spot test, which is performed by dispensing 0.5 μL labelled probe onto a marked piece of membrane. The membrane is UV cross-linked after allowing the spot to dry. The membrane is then washed in 0.1× SSC, 0.1% SDS at 65 °C for 15 min and exposed either to X-ray film or a phosphorimager cassette to check labelling. The probe can be also checked by removal of unincorporated, free nucleotides using spin columns, followed by checking the quantity of incorporated radiolabelled nucleotides by scintillation counting. However, for hybridization, it is not necessary to remove unincorporated free nucleotide.

2.6. Preparation of radiolabelled RNA markers

For small RNA Northern blotting, it is advisable to run marker RNAs adjacent to experimental RNA samples in order to correctly assess sizes of the target small RNA species. Synthetic RNAs of different lengths (for example; 18, 21 and 25 nucleotides in length) can be radiolabelled for this purpose by using T4 polynucleotide kinase (see LNA probe preparation in Section 2.4). Since RNAs are very unstable, it is important to use sterile tips and tubes and to work on ice during marker preparation. For radiolabelled markers, remove unincorporated nucleotides by filtration through a Quick Spin Column (Roche). Dilute the marker to 100 μL and load 1 μL onto a denaturing polyacrylamide gel and/or measure radioactivity to determine the optimal amount of radiolabelled marker. Alternatively, RNA marker ladders can be purchased and used according the manufacturer's instructions (for example Decade™ Markers (Ambion).

2.7. Hybridization of LNA-modified oligonucleotide probes

We routinely use hybridization solutions containing formamide for small RNA Northern blot analyses. The UV-fixed membrane is pre-hybridized at the temperature of hybridization (see below) for 30 min. Heat the labelled LNA probe for 1 min at 95 °C and cool it on ice before addition to the hybridization solution. It is recommended to replace the pre-hybridization solution at the time of probe addition.

2.7.1. The temperature of hybridization

To select the optimal temperature for hybridization we carried out a series of hybridizations applying various temperatures ranging from 37 to 60 °C. In these experiments we used total RNA extracted from *Arabidopsis thaliana* leaves and flowers and from mouse liver, as a non-specific background control. We used an LNA-modified probe (Exiqon) specific for plant miR171 which showed enhanced accumulation in flowers [7]. Replica membranes were hybridized at different temperatures overnight and washed at the temperature of hybridization two times for 10 min in 2× SSC, 0.1% SDS. The results revealed an optimal temperature of 50 °C since lower temperatures produced non-specific signals in the negative control lane (mouse liver RNA) while 60 °C reduced specific signal intensity (Fig. 1). This result indicates that 50 °C is a good starting point for efficient detection of target miRNAs while maintaining high specificity.

2.7.2. The length of hybridization

Next, we optimized the length of hybridization period. To assess the optimal period of time, we hybridized replica

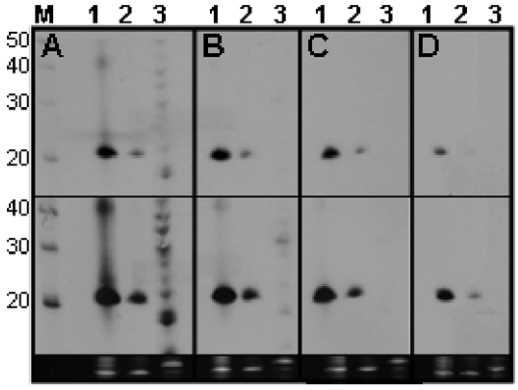

Fig. 1. The effect of temperature on hybridization efficiency. Total RNAs (10 μg/sample) from *A. thaliana* flowers (lane 1) or leaves (lane 2) and mouse liver (lane 3) were fractionated in a 12% polyacrylamide gel under denaturing conditions, blotted, and hybridized with ^{32}P-labelled miR171LNA-modified oligonucleotide probes at different temperatures. The membranes were washed two times in 2× SSC, 0.1% SDS at the temperature of hybridization for 10 min. The applied temperatures were (A) 37 °C, (B) 42 °C, (C) 50 °C and (D) 60 °C. The upper panels show 6-h and the middle panels show 12-h exposures to X-ray film. Gel loading controls are ethidium bromide-stained rRNAs (bottom panels). M denotes the RNA molecular weight marker lane.

membranes, described in the previous section, at the optimal temperature of 50 °C for 1, 2, 4, 6, and 12 h. These experiments demonstrated that a 1-h hybridization is sufficient for efficient detection of the target miRNA (Fig. 2). While a 2-h hybridization resulted in a slightly stronger signal, further extending the hybridization period did not improve signal intensity.

2.7.3. General usage of the optimal hybridization condition

To assess whether the identified optimal hybridization conditions are applicable for other probes as well, we performed hybridizations for three other plant-specific miRNAs possessing different abundances. These hybridizations showed that the previously determined optimal

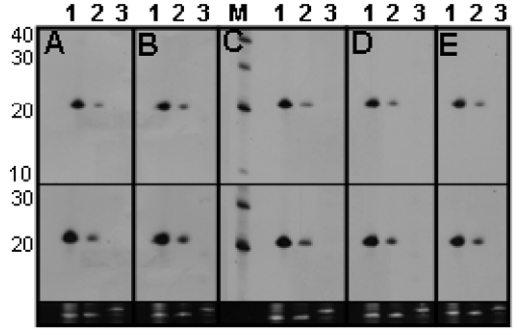

Fig. 2. The effect of the length of hybridization period on the efficiency of hybridization. Replica membranes of samples described in Fig. 1 were hybridized for (A) 1, (B) 2, (C) 4, (D) 6, and (E) 12 h at 50 °C using the same probe. Membranes were washed two times in 2× SSC, 0.1% SDS at 50 °C for 10 min. The upper panels show 6-h and the middle panels show 12-h exposures. Gel loading controls are ethidium bromide-stained rRNAs (bottom panels). M denotes the RNA molecular weight marker lane.

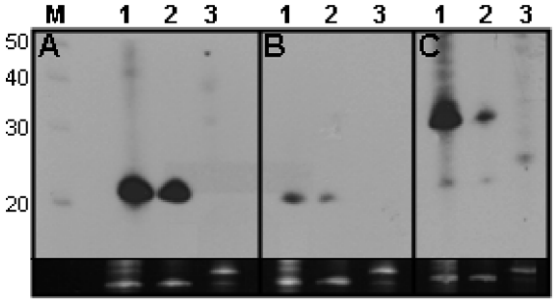

Fig. 3. Detection of different miRNAs using optimal parameters. Replica membranes of samples described in Fig. 1 were hybridized with LNA-modified oligonucleotide probes specific for (A) miR159, (B) miR160, and (C) miR164 at 50 °C for 2 h and washed two times for 10 min in 2× SSC, 0.1% SDS at the temperature of hybridization. Membranes were exposed 12 h for signal detection. Gel loading controls are ethidium bromide-stained rRNAs (bottom panels). M denotes the RNA molecular weight marker lane.

parameters are well suited for detection of other abundant (miR159) or rare (miR160) miRNAs (Fig. 3). However, in the case of miR164, we obtained additional (lanes 1 and 2) and non-specific bands (lane 3) under these conditions (see Section 2.8).

2.7.4. Washing the northern membranes

To this point, membranes were washed twice in 2× SSC, 0.1% SDS at the temperature of hybridization for 10 min. If using more than one probe in an experiment, wash the membranes in separate vessels since the extremely effective hybridization of LNA-modified probes may result in cross-hybridization in the wash solutions. Membranes may be exposed to film to check signals; however, make sure that membranes remain damp in case additional washes are required. We have not applied stringent wash conditions

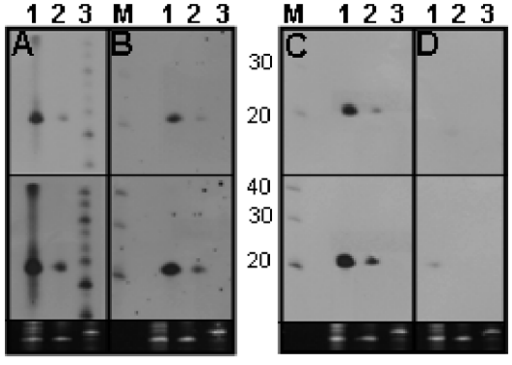

Fig. 4. Hybridization in PerfectHyb Plus using LNA-modified oligonucleotide probe. Replica membranes of samples described in Fig. 1 were hybridized with an LNA-modified oligonucleotide probe detecting miR171 in PerfectHyb Plus at (A) 50 °C and (C) 68 °C and in standard hybridization solution (see text for details) at (B) 50 °C and (D) 68 °C. Membranes were washed two times for 10 min in 2× SSC, 0.1% SDS at the temperature of hybridizations. The upper panels show 6-h and the middle panels show 12-h exposures. Gel loading controls are ethidium bromide-stained rRNAs (bottom panels). M denotes the RNA molecular weight marker lane.

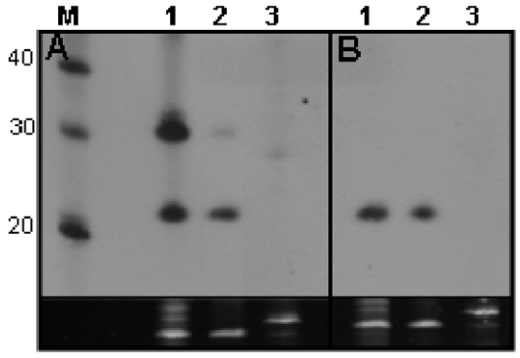

Fig. 5. Reduction of non-specific background by increased temperature and RNase A treatment. Replica membranes of samples described in Fig. 1 were hybridized with an LNA-modified oligonucleotide probe detecting miR164 in standard hybridization solution at 60 °C for 2 h. (A) The membrane was washed two times for 10 min in 2× SSC, 0.1% SDS at 60 °C and exposed for three days. (B) The membrane was washed two times for 10 min in 2× SSC, 0.1% SDS at 60 °C and treated with RNase A solution. The RNase A-treated membrane was washed again in 2× SSC, 0.1% SDS at 37 °C and exposed for three days. Gel loading controls are ethidium bromide-stained rRNAs (bottom panels). M denotes the RNA molecular weight marker lane.

in the experiments in this paper, however previously, it was demonstrated that LNA-modified oligonucleotide probes do allow the application of stringent wash conditions [1]. The stringency of wash conditions can be adjusted by gradually decreasing the salt concentration of the buffer and/or increasing the temperature. For high stringency washing of membranes hybridized with LNA-modified oligonucleotide probes, use 0.1× SSC, 0.1% SDS at 65 °C for 5 min.

2.8. Using commercially available hybridization buffers

Use of commercially available hybridization buffers, such as PerfectHyb Plus (Sigma), has recently spread throughout the world. Therefore, we wanted to test whether PerfectHyb Plus is suitable for miRNA hybridization using LNA-modified oligonucleotide probes. The previously described replica membranes were hybridized in parallel in our hybridization solution and PerfectHyb Plus at the optimal temperature (50 °C) for 2 h. At this temperature the membrane hybridized in PerfectHyb Plus showed strong non-specific background hybridizations (Fig. 4). Since the LNA-modified oligonucleotide probes exhibit unprecedented target affinity, we repeated the experiments at 68 °C as recommended by the manufacturer of PerfectHyb Plus. This hybridization experiment revealed that PerfectHyb Plus can be used for miRNA detection efficiently and specifically at this higher temperature (Fig. 4). At this temperature, our hybridization solution did not work properly, however.

2.9. Removal of non-specific, background signals

In some cases, use of LNA-modified oligonucleotide probes may result in appearance of undesired background hybridization signals. Increasing hybridization temperature can be an effective step to reduce background hybridization (Fig. 5). Application of highly stringent wash conditions can also be useful to eliminate background noise. If background hybridization signals stubbornly remain, RNase A digestion can provide an alternative method (Fig. 5). This is done by incubating the membrane, subsequently to hybridization, in 50–100 mL RNase A buffer at 37 °C for 30 min with gentle shaking. This will remove imperfect duplexes formed during hybridization. The RNase A-digested membrane will become non-reusable. Wash the membrane again for 5 min after RNase A treatment (Fig. 5). While these techniques may reduce or eliminate background hybridization, they may lower specific signal intensity and thus require prolonged exposure times for detection.

2.10. Stripping northern membranes

Membranes incubated with LNA-modified oligonucleotide probes can be stripped efficiently for reuse. Never allow membranes to dry after hybridization. Place the membrane

Table 1
Common problems and solutions

Problem	Cause	Solution
Weak or no signal	Technical problems such as poor RNA transfer etc	Use previously tested positive control samples
	Probe was not labeled efficiently	Check the probe with spot test. See Section 2.4
	The hybridization temperature was too high	Lower hybridization temperature
	Target miRNA is not present or is too low for detection	Try RNA samples originating from other tissue sources
		Increase the amount of RNA samples
Non-specific hybridization or high background	Non-specific binding of probe to non-target nucleic acids	Try higher temperature for the hybridization
		Try RNase A digestion. See Section 2.8
	Wash conditions not sufficiently stringent	Add high stringency wash step with solutions containing 0.5× SSC or 0.1× SSC
		Incresase the temperature of the washes up to 68 °C
	Exposure to film was too long	Shorten the exposure time to film

in a boiling solution of 0.1% SDS, 5 mM EDTA for 5–10 min. Avoid wrinkling the filter and do not let it dry out before complete stripping of the blot. Expose the membrane for signal detection to ensure that the probe has been stripped completely. Abundant miRNAs require longer treatment times in boiling buffer. However, fresh, non-stripped Northern blots are best for detecting miRNAs.

2.11. Troubleshooting guide

For common problems and hints for solutions see Table 1.

3. Conclusions

The technique presented here allows sensitive detection of miRNAs by Northern blot which can be extremely beneficial when small amounts of samples are available, expression levels of target miRNAs are low, or subtle discrimination of related miRNAs (differing only by a few nucleotides) is necessary. LNA modification technology has also proven its usefulness in other technologies such as *in situ* and microarray hybridizations. Commercial availability of LNA-modified probes (Exiqon, http://www.exiqon.com) and their chemical properties make them highly important and effective tools in many aspects of molecular biology. The higher cost of LNA-modified oligonucleotide probes compared to traditional DNA oligonucleotides can be compensated by the reduced time and effort required for experiments, as well as the higher chance for success. Hopefully, as the LNA-based technologies become increasingly popular and widely used, costs associated with this technology will decrease significantly.

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