Chapter 2

Detection of microRNAs in Plants by In Situ Hybridisation

Éva Várallyay and Zoltán Havelda

Abstract

MicroRNAs (miRNAs) are short, about 21 nucleotides in length, non-coding, regulatory RNA molecules representing a new layer in post-transcriptional gene expression regulation. Spatial and temporal analysis of miRNA accumulation by in situ analyses is the prerequisite of understanding the precise biological functions of miRNAs. Since miRNAs are very short molecules, their in situ analysis is technically demanding. Our method is based on the usage of highly sensitive LNA-modified oligonucleotide probes. LNA modification significantly enhances the sensitivity and specificity of miRNA detecting probes and provides relatively easy in situ miRNA detection. Here, we describe a protocol for this challenging technique step by step, in order to help every user to achieve success.

Key words: miRNA, Plant, LNA, In situ hybridisation

1. Introduction

miRNAs are key elements in biological processes; however, because of their small size, their presence and importance were discovered only with the rapid evolution of molecular biology techniques. To resolve their precise role, in situ spatial and temporal investigation of mature miRNA accumulation is needed. The technical problem that routs in the sort length of the target RNAs (21–25 nt) now seems to be solved with the use of locked nucleic acid (LNA)-modified probes (1). LNA modification in DNA oligonucleotides brings about a dramatically higher target affinity and specificity compared to a traditional DNA oligonucleotide (2). These probes have been introduced to enhance both the sensitivity and the specificity of miRNA detection by Northern blotting and in situ hybridisation (3–5). Using this technology, miRNAs can be detected relatively easily in both plant and animal tissues (5, 6). Here, we describe a detailed protocol for in situ

Tamas Dalmay (ed.), *MicroRNAs in Development: Methods and Protocols*, Methods in Molecular Biology, vol. 732,
DOI 10.1007/978-1-61779-083-6_2,

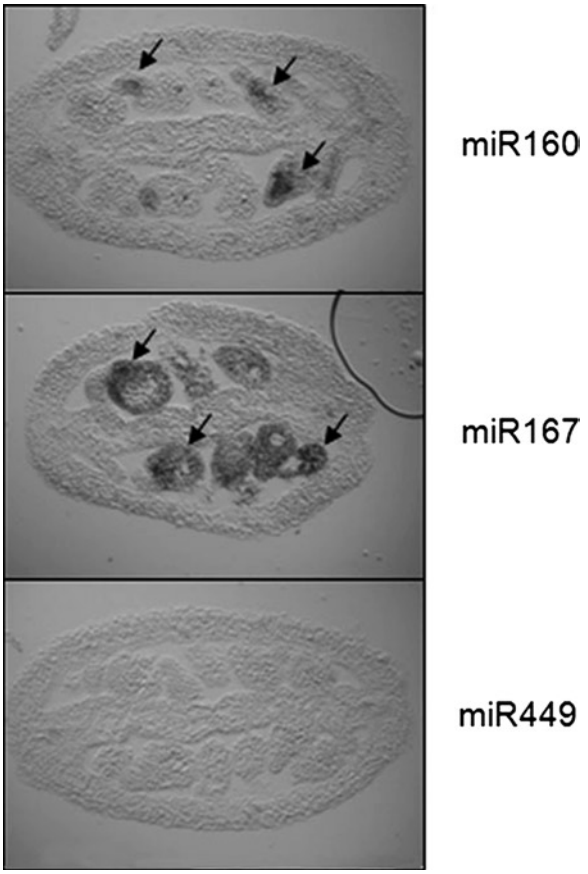

Fig. 1. Differential accumulation of miR160 and miR167 in *Arabidopsis thaliana* ovary. Longitudinal near-consecutive sections of *A. thaliana* ovary have been hybridised with 5′ and 3′ double-labelled LNA-modified oligonucleotides detecting miR160, miR167 and miR449 (chicken-specific miRNA is used as negative control). The hybridisation was carried out at 55°C overnight. *Arrows* show the accumulation of the miRNAs.

hybridisation of plant tissue sections using LNA-modified oligonucleotides (see Note 1) as probes (Fig. 1).

2. Materials

1. Alcian Blue solution: 0.25% in 3% acetic acid solution. Filtrate after preparation through a filter paper. Store at room temperature. You can use the same solution several times.
2. BCIP solution: 50 mg/ml in dimethylformamide. Store at –20°C.
3. Blocking solution I: Make it freshly every time. Dissolve 0.5% Blocking Reagent (Roche) in 1× TBS buffer at 60°C and cool down on ice.

4. Blocking solution II: Make it freshly every time. Dissolve 1% BSA in 1× TBS and add 0.3% Triton X-100.
5. DEI formamide: For hybridisation buffer, deionise with mixed bed resin (e.g., AG-501-×8 (Bio-Rad)) and store in aliquots at –20°C.
6. 100× Denhart's solution: Dissolve 10 g Ficoll400, 10 g polyvinylpyrrolidone and 10 g BSA in 500 ml sterile water. Keep aliquots frozen at –20°C.
7. Dextrane sulphate: 50% solution with water. Store in aliquots at –20°C. Note that it will dissolve only while boiling.
8. Fixative solution: Paraformaldehyde (Sigma; 4% w/v) and 0.1% Triton X-100 in phosphate-buffered saline (PBS) (see Note 2).
9. Hybridisation solution: 0.3 M NaCl, 10 mM Tris–glycine pH 6.8, 10 mM $NaHPO_4$ pH 6.8, 5 mM EDTA, 50% formamide, 10% dextran sulphate, 1× Denhardt's solution and 1 mg/ml tRNA.
10. NBT solution: 50 mg/ml in dimethylformamide. Store at –20°C.
11. 10× NTE buffer: 5 M NaCl, 100 mM Tris–glycine, pH 7.5, and 10 mM EDTA. Store at room temperature and dilute tenfold to have 1× working solution.
12. 10× PBS: 1.3 M NaCl, 0.07 M Na_2HPO_4 and 0.03 M NaH_2PO_4 (pH 6.5–7).
13. 20× Pronase buffer: 1 M Tris–glycine, pH 7.5, and 0.1 M EDTA. Store at room temperature and dilute it 20-fold to have 1× working solution.
14. Pronase solution: 40 mg/ml in water and self-digested at 37°C for 2 h to remove contaminant nuclease activities. Store in aliquots at –20°C.
15. RNase A solution: 10 mg/ml RNase A in water. Store in aliquots at –20°C.
16. 10× Saline: 8.5% w/v NaCl in water. Store at room temperature and dilute it tenfold to have 1× working solution.
17. 10× Salts buffer: 3 M NaCl, 0.1 M Tris–glycine, pH 6.8, 0.1 M $NaHPO_4$, pH 6.8, and 50 mM EDTA. Store at room temperature and dilute it tenfold to have 1× working solution.
18. 20× SSC: 3 M NaCl, 0.3 M Na_3 citrate. Store at room temperature and dilute it as indicated.
19. 10× Substrate buffer: 1 M Tris–glycine, pH 9.8, and 1 M NaCl. Store at room temperature.
20. 10× TBS buffer: 1 M Tris–glycine 1.5 M NaCl, pH 7.2.
21. tRNS solution: Prepare 100 mg/ml solution in water. Store in aliquots at –20°C.

3. Methods

In situ hybridisation of miRNAs depends upon the detection of RNA; therefore, it is very important to avoid contamination with RNases. The working environment should be clean and nuclease-free tubes, bottles, etc., should be used. The water and all aqueous solutions should be autoclaved and preferably aliquoted for single usage. (Because of its hazardous nature, we do not favour the use of diethyl pyrocarbonate treatment.)

3.1. Fixation and Embedding (Table 1)

Embedding of plant tissues in vax is a long and slow process that takes several days. After fixation of the plant tissues, we slowly saturate the tissue parts with wax to make them solid for microtom cutting. For fixation, we use formaldehyde solution, but it is not possible to change this solution directly with wax. So, first we change formaldehyde to ethanol in order to dehydrate tissues and then replace ethanol with histoclear which is a good solvent for wax. Next, we saturate the tissue parts with wax. The time of fixation is the most important step of the protocol, because if the fixation is not complete we can lose the signal and the tissue structure. On the contrary, if we over-fix the material, it will be difficult for the probe to penetrate the tissue, resulting again in the loss of signals. So, we have to optimise the fixation time for our tissue of interest. We usually refer to parameters what we have used for *Arabidopsis thaliana* or *Nicotiana benthamiana* tissues.

1. Remove samples of your interest from the plant, if it is bigger than 1 cm, then cut them into half and place them immediately into ice-cold fixative solution. We usually use 15 ml plastic tubes without the cap as container (see Note 3).
2. Keep the tubes on ice, weigh the tissue under the surface of the fixative and apply vacuum in a chamber till bubbles are formed (usually 20–30 s with the pump working) and hold the vacuum for 5–10 min. Release the vacuum very slowly. Since formaldehyde is toxic, work under a fume hood.
3. Repeat the vacuum treatment and change the fixative solution.
4. Repeat the whole procedure (2–3) until all the samples sink to the bottom of the tube (this is the indication of complete infiltration of fixative solution into the tissue samples).
5. Exchange the fixation solution when all the samples have sunk down and fix the samples at 4°C for 4 h to overnight with gentle shaking (see Note 4).
6. Degas the 1× saline solution under vacuum before usage. Wash the fixative solution with ice-cold 1× saline solution on ice.

Table 1
Fixation and embedding

Fixative solution	on ice	20–30 s	Under vacuum, several times
Fixative solution	on ice	O/N	Gentle shaking
1× saline	on ice	30 min	Degass under vacuum before usage
30% EtOH/saline	on ice	1–3 h	Degass under vacuum before usage
40% EtOH/saline	on ice	1–3 h	Degass under vacuum before usage
50% EtOH/saline	on ice	1–3 h	Degass under vacuum before usage
60% EtOH/saline	on ice	1–3 h	Degass under vacuum before usage
70% EtOH/saline	on ice	1–3 h	Degass under vacuum before usage
85% EtOH/saline	4°C	1–3 h	Degass under vacuum before usage
95% EtOH/water	4°C	1–4 h	Degass under vacuum before usage
100% EtOH	4°C	1–4 h	
100% EtOH	4°C	O/N	
100% EtOH	RT	1–2 h	
100% EtOH, 0.1% Eosin	RT	30 min	
EtOH/Histoclear = 3:1	RT	1–3 h	
EtOH/Histoclear = 1:1	RT	1–3 h	
EtOH/Histoclear = 1:3	RT	1–3 h	
Histoclear	RT	1 h	
Histoclear	RT	O/N	
Histoclear + WAX	RT	~1 day	Add wax chips several times
Histoclear + WAX	42°C	2 days	Add wax chips several times
WAX	58°C	2 days	Change half volume of the wax 2–3 times a day
WAX	58°C	2 days	Change total volume of the wax 2–3 times a day
WAX	4°C		After orientation store for unlimited time

7. Dehydrate the samples by passing them through a graded ethanol series (30, 40, 50, 60, 70 and 85% EtOH/1× saline, followed by 95% EtOH/water and finish in 100% EtOH). Degas the EtOH/1× saline-graded ethanol series under vacuum before usage. For detailed description of solution and temperature, refer to Table 1. You can interrupt the protocol and leave the samples overnight at any step after 50% EtOH/1× saline (see Note 5).

8. The next steps are performed at room temperature with gentle shaking. Replace 100%EtOH with fresh one and leave it for 1–2 h. During this period, the green colour must be eliminated. If not, repeat this step until the tissues become colourless. Then, change the EtOH with 100% EtOH containing 0.1% EosinY and agitate the tubes for 30 min (see Note 6).
9. Pass the samples through a graded histoclear series with gentle rotation (refer to Table 1. for detailed description). It is advised to carry out the Histoclear steps in glass vial or good quality plastic reaction tube, otherwise Histoclear may damage the tube. Depending on the size of the samples, every step takes 1–3 h.
10. Add one Paraplast chip to about 1 ml of histoclear and leave it to dissolve at room temperature. Once the first chips have dissolved add other ones, and so on, until the chips will not dissolve any more. At this stage, transfer the samples to 42°C and saturate the histoclear by adding more and more Paraplast chip till saturation.
11. Put samples at 58°C and overlay it with freshly melted Paraplast (avoid heating Paraplast over 58°C). Try to form a cap of melted wax on the top of solution to avoid heat stress of the samples. Change half of the volume for freshly melted Paraplast and leave at 58°C overnight.
12. Change half of the wax twice a day to freshly melted wax for 2 days. (Decant gently the old Paraplast and pour in fresh melted Paraplast.) Leave the tubes open to allow Histoclear to evaporate.
13. Try to change the total volume to freshly melted wax for another 2 days.
14. Prior to sectioning, the tissue samples must be solidified in a wax block. Put the plastic or metal moulds on a hot plate at 60°C, shake up the samples and pour them out into the mould. Ideally, about 10–12 samples should be transferred into one mould. Arrange the samples in rows leaving sufficient space between them allowing the cutting out of single blocks containing one or a few samples. Put the samples on ice or at 4°C and allow them to harden. Apply the tissue block on a holder compatible with the microtome.

3.2. Sectioning

Good sections are very important for nice results. It is worthwhile to practise it with somebody who has done it before. You will need patience and practise and correctly embedded samples to do perfect sections.

1. Make tissue sections (8–20 μm) using a retracting rotary microtome. Trim the block so that the upper and lower faces are parallel. Repeated sectioning leads to the formation of

ribbon of sections. Trimming a trapezoid shape block helps the identification of a single section in the ribbon.

2. Mount the sections onto poly-L-lysine-coated pre-prepared slides. Wax sections need to be stretched before adhesion to the glass slide. Sections should be put onto a layer of degassed water on a slide held on a warmed hotplate (40–42°C). Once the section has stretched, drain away the excess water and leave the slide to dry overnight 40–42°C.

3.3. LNA Probe Preparation and Probe Checking

LNA-modified oligonucleotide-based probes can be purchased from Exiqon (Denmark; www.exiqon.com) and its labelling depends on the required sensitivity. *Arabidopsis thaliana* tissue sections respond less efficiently in in situ experiments than other plant tissues (for example *N. benthamiana*). While the 3′ end-labelled probes (using the DIG oligonucleotide 3′-end labelling kit (Roche)) work well in *N. benthamiana*, they do not provide reliable signals in *A. thaliana*. To achieve good signals in *A. thaliana* in situ experiments, it is recommended to order 5′ chemically DIG-labelled LNA-modified probe and also label at the 3′ end, producing a double-labelled probe. Alternatively, double-labelled probes are also possible to order. It is very important to use a similarly labelled negative control (for example, an animal-specific miRNA) during the experiments to monitor background hybridisation.

1. Label 50 pmol LNA-modified oligonucleotide or 5′ DIG-labelled LNA-modified oligonucleotide in 10 µl volume using the DIG oligonucleotide 3′-end labelling kit (Roche), according to the manufacturer's instructions. It is not necessary to purify the probe after labelling.
2. Remove 0.2 µl (for probe checking) from the 10 µl reaction and add 10 µl deionised formamide.
3. It is sensible to check the quality of the labelled probe prior to usage. To do this, spot 0.2 µl aliquot (and the labelled control oligonucleotide provided by the kit) on a piece of membrane and cross-link with UV light.
4. Put the membrane in 1× TBS buffer for 2 min and incubate the membrane in TBS buffer containing 5% of powdered milk for 10 min (Blocking). Add anti-DIG-alkaline phosphatase and Fab fragments (1:2,000) and hybridise with gentle shaking for 30 min.
5. Wash at least three times in 1× TBS buffer for 5 min each and transfer to 1× SB for 2 min (washing).
6. Develop the colour reaction by adding NBT and BCIP (add 3 µl NBT and 3 µl BCIP solution into 1 ml 1× SB). Stop the reaction by rinsing the membrane with water and dry the membrane.

3.4. Slide Preparation for Hybridisation (Table 2)

To make the tissue sections on the slide accessible for solvents, you have to remove wax. During the protocol, you have to move the slides together between different solvents. You can decide to move them individually (it is very difficult to handle more than 10 slides with this method) or you can put them in a slide holder. (This is more convenient and faster. With this method, you can move up to 20 slides together.)

1. Transfer the slides into histoclear at room temperature and incubate for 10 min. For the first treatment, the histoclear can be reused from the second treatment of a previous experiment. Repeat using fresh histoclear.
2. Transfer slides into 100% EtOH (can be reused from the previous experiment before) for 5 min. Repeat once using fresh 100% EtOH.
3. Hydrate the samples by passing them through a graded ethanol series (95, 85, 75, 50, 30% EtOH/1× saline) for 2 min at each step. For a detailed description of solutions and temperature, refer to Table 2. This ethanol series can be reused for several times in the subsequent experiments, and can be reused directly in the reverse order in this experiment at a later stage (see point 6). Finish by washing the sides in 1× saline for 2 min.
4. Equilibrate the slides in 1× pronase buffer for 2 min at 37°C. Incubate the slides in pronase solution (containing 10 µg/ml pronase) for 10 min at 37°C. Incubate the slides in 0.2% glycine in 1× PBS for 2 min and then wash them in 1× PBS for 2 min.
5. Postfix in fixative solution (in a fume hood) for 30 min. Wash the slides twice in 1× PBS for 2 min each.
6. To eliminate background reaction of the hybridisation probe, due to electrostatic binding, amino groups on the section should be acetylated using an acetic anhydride treatment (1 ml acetic anhydride in 200 ml 0.1 *M* triethanolamine-HCL, pH 8.0). Incubate the slides in buffered acetic anhydride for 10 min at room temperature (see Note 7).
7. Wash the slides once in 1× PBS for 2 min. Dip the slides in fresh 1× saline solution for 2 min and dehydrate through a graded ethanol/saline series (30, 50, 75, 85, 95% EtOH/1× saline) for 2 min at each step. If you have saved the ethanol series at point 3, you can reuse it now.
8. Transfer the slides to 100% EtOH.

Now the slides are ready for hybridisation. You can stop here and keep the slides safely in EtOH for hours, but in this case put them to 4°C.

Table 2
Slide preparation for hybridisation

(a) Removal of the wax from the tissues			
Histoclear	10 min	You can use a used one, but waste it after usage	
Histoclear	10 min	Use fresh one and keep it for reuse once	
100% EtOH	5 min	You can use a used one, but waste it after usage	
100% EtOH	5 min	Use fresh one and keep it for reuse	
95% EtOH/saline	2 min		
85% EtOH/saline	2 min		
75% EtOH/saline	2 min		
50% EtOH/saline	2 min		
30% EtOH/saline	2 min		
100% 1× saline	2 min		
All these reactions should be made at room temperature			
(b) Treatment with pronase and elimination of background signals			
1× Pronase buffer	37°C	2 min	
Pronase in pronase buffer	37°C	10 min	50 µl 40 mg/ml Pronase solution in 200 ml pronase buffer
Glicin	RT	2 min	0.2% Glycine solution in 1× PBS
PBS	RT	2 min	
Fixative solution	RT	30 min	
PBS	RT	2 min	
PBS	RT	2 min	
Acetic anhydride	RT	10 min	For 200 ml solution add 1 ml acetic anhydride to 2.5 ml triethanolamine + 1 ml ccHCl to sterile water just before using
PBS	RT	2 min	
100% 1× saline	RT	2 min	
30% EtOH/saline	RT	2 min	
50% EtOH/saline	RT	2 min	
75% EtOH/saline	RT	2 min	
85% EtOH/saline	RT	2 min	
95% EtOH/saline	RT	2 min	
100% EtOH	RT	2 min	If you leave the slides for longer period in EtOH, put to 4°C

3.5. Hybridisation

1. Prepare hybridisation solution about 100–200 µl per slide depending on the number and size of the sections. For 1 ml hybridisation solution, add 100 µl 10× salts buffer, 500 µl deionised formamide, 250 µl 50% dextran sulphate, 10 µl 100 mg/ml tRNA, 10 µl 100× Denhardt's solution and water (see Note 8).
2. Add 1–5 µl labelled LNA probe (in 50% formamide) per slide to the hybridisation solution. Vortex well, centrifuge to eliminate bubbles and keep at the temperature of hybridisation (50–60°C) (see Note 9).

3. Put one slide on a hot plate at 50°C and allow it to dry. Do not over dry your sections. Apply the hybridisation solution with probe as a band along the middle of the slide and cover it. Put the slide in a closed environment saturated with 50% formamide/2× SSC pre-warmed to the temperature of hybridisation. Prepare a plastic box with 50% formamide/2× SSC at bottom. Place the slides on a horizontal support inside the plastic box (see Note 10).
4. Repeat with every slide individually. Close the box, seal with clingfilm and incubate the slides at the temperature of hybridisation overnight. Prepare washing solution in excess (0.2× SSC) and place at the temperature of hybridisation.

3.6. Washing and Signal Detection (Table 3a, b)

1. Start washing at 50–60°C (the same temperature as hybridisation) in 0.2× SSC. Put slides into washing solution and carefully remove the cover slips or parafilms. Rinse the slides having different probes separately several times to avoid cross-contamination of probes and wash them for 30 min. Wash the slides twice at the temperature of hybridisation for 1 h each (see Note 11).
2. Immerse the slides in 1× NTE buffer pre-warmed to 37°C, repeat in fresh buffer 5 min each. Incubate the slides in NTE containing 20 µg/ml RNase A at 37°C for 30 min to remove background hybridisation.
3. Rinse the slides in 1× NTE for 5 min at room temperature and transfer them to washing solution (0.2× SSC) for 1 h at the temperature of hybridisation. Dip the slides into 1× SSC for 2 min then into 1× TBS twice for 5 min each time. Slides are now ready for the detection step.
4. Incubate the slides in Blocking solution I for 30 min. Transfer the slides into Blocking solution II and incubate for 30 min (see Note 12).
5. Add anti-DIG-alkaline phosphatase, Fab fragments (Roche) (1:2,000) to the required amount of Blocking solution II. (0.5 ml per slide). Place the slides individually on a horizontal support and put them into moist plastic or glass chamber on a tray. Apply the antibody solution onto the slides and incubate for 90 min at room temperature.
6. Stop the reaction by washing the slides (transferred back to the rack) at least five times in excess 1× TBS for 5 min. Equilibrate the slides in 1× SB for 5 min.
7. To develop the colour reaction, apply NBT and BCIP containing 1× SB (add 30 µl NBT and 30 µl BCIP solution to 10 ml 1× SB) on the slide. Remove the slides one by one from 1× SB and immediately apply the substrate solution because after drying it can be difficult to spread the liquid. Put the

Table 3
Washing and signal detection

(a) Washing			
0.2× SSC	Temperature of hybridisation	30 min	Wash slides with different LNA probes separately
0.2× SSC	Temperature of hybridisation	1 h	
0.2× SSC	Temperature of hybridisation	1 h	
NTE	37°C	5 min	
NTE	37°C	5 min	
RNase A in NTE	37°C	30 min	400 µl 10 mg/ml RNase A solution in 200 ml NTE
NTE	RT	5 min	
0.2× SSC	Temperature of hybridisation	1 h	
1× SSC	RT	2 min	
TBS	RT	5 min	
TBS	RT	5 min	
(b) Blocking, hibridisation with anti-DIG and staining			
Blocking reagent/TBS	30 min		1 g blocking reagent/200 ml 1× TBS
1% BSA, 0.3% Triton X-100/TBS	30 min		2 g BSA/600 µl Triton X-100/in 200 ml TBS
1% BSA, 0.3% Triton X-100/TBS + Anti-DIG 1:2,000	90 min		Slides individually 500 µl/slide
TBS	5 min		
TBS	5 min		
TBS	5 min		
TBS	5 min		
TBS	5 min		
Substrate buffer	5 min		Dilute 20 ml 10× SB + 4 ml 2.5 M $MgCl_2$ to 200 ml with distilled water
NBT/BCIP/$MgCl_2$/substrate buffer	2–24 h and up		30 µl NBT and 33 µl BCIP in10 ml in $MgCl_2$/SB Slides individually 0.8–1 ml/slide
All these reactions should be made at room temperature			

(continued)

Table 3
(continued)

(c) Final washing and background staining		
Distilled water	2–5 min	
40% EtOH/water	2–5 min	
70% EtOH/water	2–5 min	
100% EtOH/water	2–5 min	
70% EtOH/water	2–5 min	
40% EtOH/water	2–5 min	
Distilled water	2–5 min	
Alcian Blue	5–15 min	99 ml water + 1 ml acetic acid +0.25 g Alcian blue
Distilled water	2 min	
All these reactions should be made at room temperature with gentle shaking		

slides into a moisture chamber and cover them individually with about 0.8–1 ml of substrate solution. Cover the moisture chamber to keep the slides dark, until staining develops.

8. Monitor the signal development for 2–24 h. For rare miRNA targets, you may need longer signal development time. Stop the reaction at the desired signal intensity by rinsing the slides in water (see Note 13).

3.7. Final Washing and Background Staining (Table 3c)

Before doing this final washing step, investigate your samples in order to decide if it is necessary or not. Compare the slides with negative control and if you detect high level of background hybridisation, then you need to wash your slides. If you don't need final washing, go directly to the counterstaining step (point 2).

1. Wash the slides in a graded EtOH series for 2–5 min (depending on the intensity of the signal and background) at each step (40, 70 and 100% EtOH). Repeat the process in reverse direction. For a detailed description of solution and temperature, refer to Table 3.
2. Counter-stain the sections by dipping the slides for 5–15 min in 0.25% Alcian blue in 3% acetic acid. The slides should be monitored for the intensity of staining (tissue having no hybridisation signals should show a faint blue staining). Rinse the slides in water and air-dry.
3. Cover the slides with cover slips using mounting solution (DPX), about 100–200 µl per slide. Leave the slides to dry for a few hours. Now the sections are ready for data recording using a standard light microscope (see Note 14).

4. Notes

1. LNA-modified oligonucleotide probes detecting miRNAs can be ordered from Exiqon (http://www.exiqon.com) and a website for probe design is also available (http://lnatools.com).
2. It should be prepared in a screw-top bottle (e.g., Duran type) under a fumehood. Take 100 ml 1× PBS, pH = 6.5–7 and using a solution of NaOH adjust pH = 11. Measure 4 g paraformaldehyde to the bottle, fill it with the 1× PBS and heat the solution to 60°C. Shake vigorously for about 30 s and release the pressure every 5–10 s. Leave to settle. Most of the paraformaldehyde should be dissolved, though the solution may still be slightly cloudy. Cool it on ice. Use H_2SO_4 to adjust the pH back down to 7 (do not use HCl as this releases a carcinogen). Add 0.1 ml of Triton X-100 to the solution. You can

prepare larger volume solution and store it at –20°C in aliquots. Once you have thawed an aliquot do not freeze back.

3. The ratio between the sample and the fixative solution is crucial. In a 15-ml tube, in 8–10 ml fixative put no more than 10 pieces of 1 × 1 cm tissue from bigger leaves, 20–30 *Arabidopsis* seedling, 20–30 *Arabidopsis* siliques and 20 *Arabidopsis* inflorescens. For samples such as silique, make a cut at some part to make it possible to the fixative to get inside.
4. The fixation time strongly depends on the size and the tissue type of the samples. Larger and compact tissue samples usually require longer fixation (for example, 4h for *Arabidopsis* seedling, 6–8 h *Arabidopsis* inflorescence and 12–14 h for *Arabidopsis* siliques); however, over fixation can reduce the hybridisation signal.
5. Depending on the size of the samples, every step takes 1–3 h. For *Arabidopsis* leaves, inflorescence the above protocol is perfect. However, for more compact tissues such as *Nicotiana* leaves and seeds, it is more sensible to use less steps, but for longer incubation time (50–70–85–95%EtOH/1× saline for up to 3 h every step).
6. Staining with eosin is important as it will make orientation and sectioning easy. Without eosin staining, you won't see the tissue in the wax. Do not over stain your samples, as it will be difficult to wash out this stain from the tissue sections and it may interfere with the signal detection.
7. To prepare 200 ml 0.1 *M* triethanolamine-HCL buffer, pH 8.0, add 2.5 ml triethanolamine and 1 ml HCl to water and stir. Add 1 ml acetic anhydride to 200 ml triethanolamine buffer and stir vigorously (work under fume hood). Since the acetic anhydride is very unstable in water, it has to be added just before using.
8. The volume of the probe usually does not alter significantly the concentration of hybridisation solution. As this hybridisation buffer is very viscous, prepare a bit more than the desired volume as you can lose quite a lot of solution with pipetting.
9. The temperature of hybridisation strongly depends on the nature of the particular probe. 55°C is a good starting temperature. If the probe tends to give background hybridisation, then increase the temperature of hybridisation to 60°C and in parallel increase also the temperature of washing. If no signal is detected, then lower the temperature of hybridisation and washing to 50°C.
10. The slide must be covered here, but with a traditional cover slip it seemed very difficult for us. As an alternative, cut Parafilm precisely into a coverslip size and with the help of forceps, put the Parafilm on the slide from one side of the

slide slowly. With this method, formation of bubbles under the cover slip can be prevented. It is very important to prevent the bubble formation because hybridisation will not occur in the presence of bubbles.

11. Be very cautious with the removal of the cover slips as you can harm the tissue sections easily. The easiest way to this is to put the slide vertically into washing buffer and wait a bit until the cover slips fall off.
12. Blocking reagent is difficult to dissolve in 1× TBS at room temperature. You have to heat it, but before usage, put the solution on ice because ice-cold Blocking solution gives much better result than room temperature solution.
13. If you have to wait for signal development for a longer period, change the NBT/BCIP substrate solution twice a day. We usually do not cover the slides during stain development, so it is easy to do this; just drain off the used stain and apply new one on the slide. Although it is possible to check the staining with the stains on, in this case, you have to cover the slides with a cover slip.
14. After using DPX, it is very difficult to remove the cover slips (you can remove them by putting the slides in histoclear). Use 50% glycerol if you want to further wash your slides.

Acknowledgments

This work was supported by a grant from the Hungarian Scientific Research Fund (OTKA K78351). VÉ is a recipient of Bolyai Janos Fellowship.

References

1. Kauppinen, S., Vester, B., and Wengel, J. (2006) Locked nucleic acid: high-affinity targeting of complementary RNA for RNomics. *Handb Exp Pharmacol* **173**, 405–22.
2. Valoczi, A., Hornyik, C., Varga, N., Burgyan, J., Kauppinen, S., and Havelda, Z. (2004) Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. *Nucleic Acids Res* **32**, e175.
3. Varallyay, E., Burgyan, J., and Havelda, Z. (2007) Detection of microRNAs by Northern blot analyses using LNA probes. *Methods* **43**, 140–45.
4. Varallyay, E., Burgyan, J., and Havelda, Z. (2008) MicroRNA detection by northern blotting using locked nucleic acid probes. *Nature Protocols* **3**, 190–96.
5. Valoczi, A., Varallyay, E., Kauppinen, S., Burgyan, J., and Havelda, Z. (2006) Spatio-temporal accumulation of microRNAs is highly coordinated in developing plant tissues. *Plant Journal* **47**, 140–51.
6. Kloosterman, W. P., Wienholds, E., de Bruijn, E., Kauppinen, S., and Plasterk, R. H. (2006) In situ detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. *Nat Methods* **3**, 27–9.

http://www.springer.com/978-1-61779-082-9

MicroRNAs in Development
Methods and Protocols
Dalmay, T. (Ed.)
2011, X, 200 p., Hardcover
ISBN: 978-1-61779-082-9
A product of Humana Press