Chapter 7

Small RNA In Situ Hybridizations on Sections of *Arabidopsis* Embryos

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Abstract

Small RNAs mediate posttranscriptional gene silencing in plants and animals. This often occurs in specific cell or tissue types and can be necessary for their differentiation. Determining small RNA (sRNA) localization patterns at cellular resolution can therefore provide information on the corresponding gene regulatory processes they are involved in. Recent improvements with in situ hybridization methods have allowed them to be applied to sRNAs. Here we describe an in situ hybridization protocol to detect sRNAs from sections of early staged *Arabidopsis thaliana* (*Arabidopsis*) embryos.

Key words In situ hybridization, Small RNA, microRNA, Plant development, Embryo, *Arabidopsis*

1 Introduction

RNA in situ hybridization is a technique that utilizes antisense oligonucleotide probes to detect complementary RNAs in a tissue of interest. This enables the characterization of RNA localization patterns and thus yields insights into their functions. The incorporation of locked nucleic acids (LNAs) in oligonucleotide probes increases hybridization probe affinity and thermal stability of the probe–target RNA duplex. Consequentially, the length of the probe required for stable target RNA duplex formation can be reduced facilitating the detection of small RNAs (sRNAs) from various species and tissue types [1–8]. An additional fixation step uses 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to immobilize the 5′ monophosphates of sRNAs to the specimen's protein matrix. This enhanced the sensitivity and robustness of sRNA in situ hybridization methods [9, 10].

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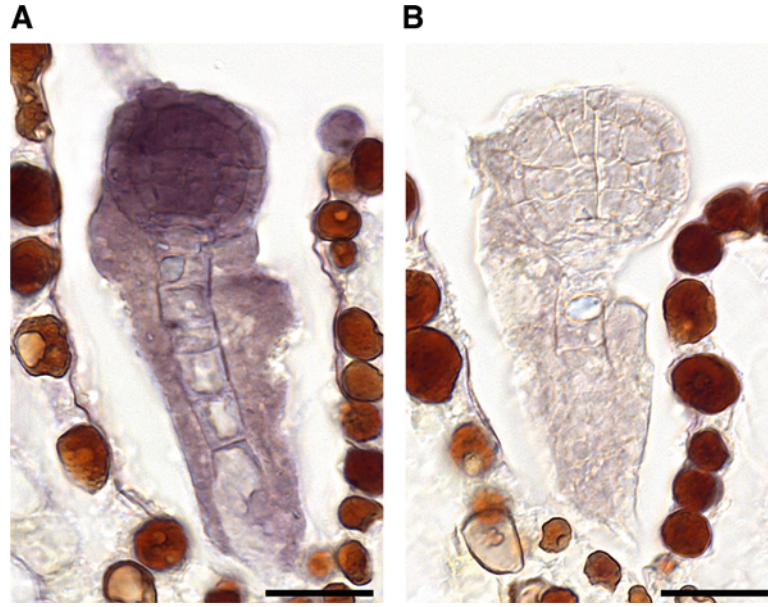

Fig. 1 Example images of small RNA in situ hybridizations on sectioned *Arabidopsis* embryos. Small RNA in situ hybridizations were performed with LNA-containing and dual DIG-labeled probes antisense to either the plant-specific miR156 (**a**) or animal-specific miR124 (**b**; negative control). Scale bars represent 20 μm. Oligonucleotide probe sequences and modifications were designed as described in [10]

Here, we present a detailed protocol for sRNA in situ hybridization on sections of *Arabidopsis* embryos. This protocol is based on previous mRNA and sRNA in situ protocols [3, 10–13]. Paraffin-embedded siliques are sectioned allowing access to young embryos, and hybridization is carried out with LNA-containing probes that are dual end-labeled with digoxigenin (DIG). Once probes are designed and prepared, the experiment takes 7 days to complete: tissue fixation and dehydration (days 1–3), clearing, embedding and sectioning (days 3–5), proteinase K digestion, EDC fixation and probe hybridization (days 5–6), washing and antibody reaction (day 6), and colorimetric reaction and mounting (days 6–7). Although this protocol was optimized for sectioned *Arabidopsis* embryos, it can be adapted to other tissue types with modifications as noted. Using this method, we were able to visualize the expression domain of miR156 from early *Arabidopsis* embryos (Fig. 1).

2 Materials

2.1 Reagents and Solutions

Solutions 1–12 must be made from RNase-free components.

1. DEPC-treated water: in the fume hood, add DEPC to a final concentration of 0.1% to deionized water, mix on a magnetic

stirrer overnight, and then deactivate DEPC by autoclaving the next morning (*see* **Note 1**).

2. Formaldehyde-acetic acid-alcohol fixative (FAA; 50 ml): in the fume hood, add 17.5 ml DEPC-treated water, 5 ml 37% formaldehyde, 2.5 ml acetic acid and 25 ml 100% ethanol to 50 ml Falcon tube, mix and place on ice.
3. Eosin Y (0.1%): Dissolve 50 mg Eosin Y in 50 ml of 95% ethanol.
4. TE (10×): 100 mM Tris, 10 mM EDTA, pH 7.5 (*see* **Note 2**).
5. Proteinase K buffer (5×): 500 mM Tris, 250 mM EDTA in Milli-Q water, pH 8.
6. Proteinase K (10 mg/ml): Dissolve 100 mg in 100 ml 1× Proteinase K buffer, make single-use aliquots and store at −20 °C.
7. Methylimidazole-NaCl: 0.13 M methylimidazole, 300 mM NaCl, pH 8.
8. Phosphate-buffered saline (PBS; 10×): 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4.
9. EDC solution: 0.16 M *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride in Methylimidazole-NaCl.
10. Glycine (10×): 20 mg/ml glycine, 0.15 M NaCl, 3 mM NaH_2PO_4, 7 mM Na_2HPO_4, pH 7.
11. Hybridization Salts (10×): 3 M NaCl, 0.1 M Tris–HCl, 0.1 M Na_2HPO_4, 0.05 M EDTA, disodium salt dihydrate, pH 6.8. Make single-use aliquots and store at −20 °C.
12. Dextran sulfate and Denhardt's solution. Make single-use aliquots and store at 4 °C.
13. tRNA: 50 mg/ml tRNA in DEPC-treated water, make single-use aliquots and store at −20 °C (*see* **Note 3**).
14. SSC (20×): 3 M NaCl, 0.3 M trisodium citrate.
15. Maleic acid, sodium chloride buffer (MN buffer; 10×): 1 M maleic acid, 1.5 M NaCl, pH 7.5 (*see* **Note 4**).
16. Blocking reagent solution (10×): Dissolve 10 g of blocking reagent per liter 1× MN buffer and store at 4 °C.
17. Tris-buffered saline (TBS; 10×): 1 M Tris, 1.5 M NaCl, 5 mM $MgCl_2$, pH 9.0.
18. BSA solution: Dissolve 6 g BSA with 1.6 ml Triton X-100, 60 ml 10× TBS and 540 ml Milli-Q water.
19. TNM5 buffer (1×): 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.0.
20. TNP buffer (1×): 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, 10% PVA, pH 9.0.

21. RNaseZap (ThermoFisher) or similar RNase decontamination solution.
22. HistoClear II or xylenes.
23. Paraplast Plus (required if manually embedding specimen).
24. EDC (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride.
25. Ultrapure (GC ≥99.5%) and standard formamide. Make single-use aliquots of ultrapure formamide and store at −20 °C.
26. DIG Nucleic Acid Detection Kit including Blocking reagent, NBT/BCIP stock solution and Anti-DIG-AP conjugate antibody (Roche).
27. Blocking reagent.
28. Anti-DIG-AP conjugate antibody.
29. NBT/BCIP Stock Solution (NBT/BCIP Stock Solution). Make single-use aliquots and store at −4 °C.
30. Aqua Poly/Mount (Polysciences).

2.2 Equipment

1. 50 ml conical tubes (e.g., Falcon).
2. 20 ml glass scintillation vials.
3. Vacuum pump or access.
4. Automatic tissue embedding system (e.g., LOGOS Microwave Hybrid Tissue Processor from Milestone Medical) (optional).
5. Slide warming table.
6. Aluminum weighing dishes.
7. Insect pins.
8. Microtome.
9. Paintbrushes or cotton-tipped applicators.
10. Razor blades.
11. Hot plate.
12. Water bath.
13. Superfrost™ Ultra Plus Adhesion Slides.
14. Temperature-controlled incubators.
15. Staining dishes (~20–25) and holders (~5) (e.g., 10-slide dishes from Wheaton).
16. HybriSlip™ Hybridization Covers (24 mm × 60 mm × 0.25 mm; ThermoFisher).
17. Tupperware or similar plastic boxes with airtight seals (to use as a humidified chamber during hybridization).
18. Benchtop shaker.
19. Shallow plastic trays (e.g., 20 cm × 30 cm).

20. Slide mailers.

3 Methods

3.1 Probe Design

When designing probes, first place LNA modifications in central positions of the probe and then progressively add LNAs in evenly spaced positions along nonterminal positions of the probe until the desired RNA melting temperature between 80 and 90 °C is reached (*see* **Note 5**).

3.2 Tissue Fixation and Dehydration (Days 1–3)

1. Remove siliques from plant and gently slice both sides along the replum with a scalpel or needle (*see* **Note 6**). Place 15–20 siliques in 10 ml ice-cold FAA within a glass scintillation vial. Then vacuum infiltrate by pulling and releasing the vacuum as slowly as possible. Repeat it a few times until all siliques sink to the bottom of the vial. Gently tap desiccator occasionally to release air bubbles. Place vial at 4 °C for 10–12 h (*see* **Note 7**).
2. Remove FAA from siliques and wash with 50% ethanol.
3. Add fresh 50% ethanol and incubate for 30 min at room temperature. Repeat with 65% ethanol, 80% ethanol and 95% ethanol (*see* **Note 8**).
4. Remove 95% ethanol and replace with 0.1% Eosin Y. Incubate overnight at 4 °C (*see* **Note 9**).
5. Remove 0.1% Eosin Y and replace with 100% ethanol. Incubate for 1 h at room temperature.
6. Exchange 100% ethanol with fresh 100% ethanol and incubate for 30 min. Repeat 1× (*see* **Note 10**).

3.3 Clearing, Sectioning and Embedding (Days 3–5)

1. Samples can be cleared and embedded using an automatic tissue processor (e.g., we use LOGOS Microwave Hybrid Tissue Processor from Milestone Medical using the standard overnight cycle) (*see* **Note 11**).
2. Remove cassettes containing siliques from the tissue processor and place on a hot plate. Alternatively if embedding was done by hand, prepare paraffin blocks by pouring warm embedded material into an aluminum weighing dish on the hot end of slide warming table. Use insect pins to orient siliques so that they are in the same orientation. Carefully move aluminum dish to cooler part of the hot plate and rearrange as necessary. Let blocks harden for at least 1 h and store at 4 °C until needed.
3. Using a standard microtome, cut paraffin-embedded siliques into 8–10 μm sections (*see* **Note 12**). Cut ribbons into approximately 2.5 cm strips with razor blades and carefully float strips in water bath filled with 42 °C fresh deionized water for approximately 1 min. Then mount each ribbon on a glass slide by placing the slide underneath the section and carefully

lifting up to capture the section on the slide (*see* **Note 13**). Place vertically for a couple of minutes to remove excess water.

4. Immediately place mounted slides on a hot plate (or in an incubator) set to 45–50 °C overnight to bake the sections onto the slides.

3.4 Proteinase K Digestion, EDC Fixation and Probe Hybridization (Days 5–6)

Before starting the experiment bake all glassware (i.e., staining dishes, slides holders) at ≥180 °C. Be careful not to heat up or cool down too quickly as this will crack the dishes. Clean all plastic tools with RNaseZAP and rinse with fresh water (forceps, brushes, plastic boxes, etc.). Clean bench with RNaseZAP and always wear gloves while handling samples. Before the probe hybridization step only use DEPC-treated water and DEPC-treated PBS or solutions made with them (*see* **Note 14**).

3.4.1 Dewaxing

1. Equilibrate slides to room temperature (*see* **Note 15**).
2. Put slides in a staining dish slide holder and fill with clearing reagent (i.e., HistoClear or xylenes (*see* **Note 16**)). Incubate for 10 min at room temperature. Dip slides up and down a few times during the incubation. Repeat 1× with fresh clearing reagent.

3.4.2 Hydration

1. Transfer slides from clearing reagent to a staining dish containing 100% ethanol. Dip slides up and down 15×. Incubate for 5 min to remove clearing reagent. Repeat 1×.
2. Process the slides through 95%, 85%, 70%, 50%, 30% and 15% ethanol. For each ethanol series, dip slides up and down 15× and incubate for 2 min (*see* **Notes 17** and **18**).
3. Incubate in 1× TE for 2 min. Repeat 1× with fresh 1× TE.

3.4.3 Proteinase K Digestion

1. Incubate slides for 10 min in 1× proteinase K buffer (without proteinase K) at room temperature. Dip slides up and down a few times to equilibrate the sections (*see* **Note 19**).
2. Add proteinase K to the prewarmed proteinase K buffer to a final concentration of 1 μg/ml. Transfer slides to staining dish containing proteinase K buffer (with proteinase K), and incubate at 37 °C for 30 min (*see* **Note 20**).
3. Transfer slides to a staining dish containing 1× glycine. Dip up and down a few times to rinse off the proteinase K and incubate for 2 min at room temperature.

3.4.4 EDC Fixation

1. Transfer slides to a staining dish containing 1× PBS, dip up and down a few times to rinse off glycine and incubate for 2 min. Repeat 1×.

2. Transfer slides to a staining dish containing freshly prepared methylimidazole-NaCl and incubate for 10 min at room temperature. Repeat 1×.
3. Transfer slides to EDC solution and incubate for 2 h at 60 °C.
4. Transfer slides to 1× PBS, dip up and down a few times and incubate for 5 min. Repeat 2×.

3.4.5 Dehydration

1. Transfer slides to a staining dish containing 15% ethanol, dip up and down a few times, and incubate at room temperature for 2 min. Repeat for 30%, 50%, 70%, 85%, 95%, and 100% ethanol. Dip slides up and down 15× and incubate for 2 min in each ethanol solution (*see* **Note 21**).
2. Let slides air-dry on bench with sections facing up for 2 h.

3.4.6 Probe Hybridization

Preheat an incubator to the preferred hybridization temperature. Prepare a humidified box for slides. We use airtight sealed plastic boxes filled with paper towels soaked in 50% formamide (standard grade) at the bottom, layered with glass pipettes on the top (Fig. 2). The pipettes prevent slides from coming into contact with the paper towels (*see* **Note 22**).

1. Prepare Hybridization Mix. Pipet the reagents in the following order to prepare 2 ml: 150 µl nuclease-free water, 250 ml 10× hybridization salts, 1 ml ultrapure and ice-cold formamide, 0.5 ml 50% dextran sulfate, 50 µl Denhardt's solution, and 50 µl 50 mg/ml tRNA. Mix by pipetting up and down slowly to avoid air bubbles (*see* **Notes 23** and **24**).

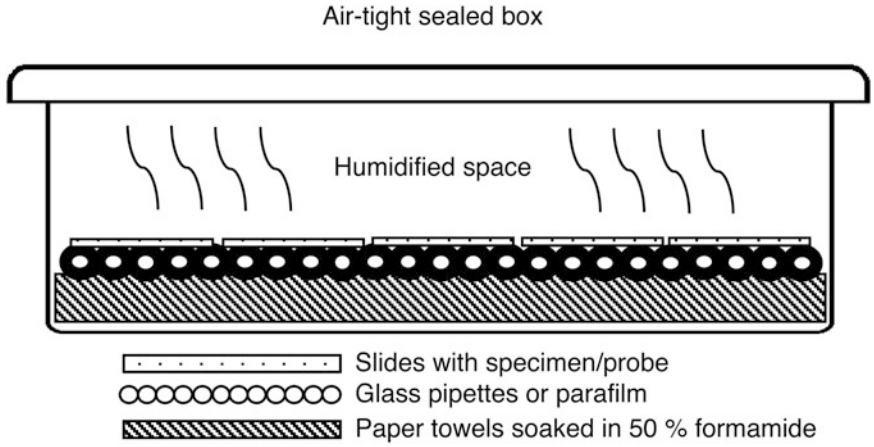

Fig. 2 Assembly of probe hybridization chamber. In an airtight sealed plastic box (e.g., Tupperware), layer paper towels soaked in 50% formamide on the bottom to create a humidified chamber and place glass pipettes (or Parafilm) on top of paper towels to create a barrier between the formamide and slides. Cover chamber with an airtight lid and prewarm in an incubator to the desired hybridization temperature. Remove from incubator, add slides with specimen/probe, cover chamber, and place back in incubator for 10–12 h

2. Prepare LNA probes: Add 160 μl of hybridization solution to separate Eppendorf tubes (one for each slide to be hybridized). Add appropriate amount of each probe to individual 200 μl PCR strip tubes and bring up to 40 μl with 50% formamide (*see* **Note 25**). Incubate at 80 °C for 2 min and then 4 °C in thermal cycler. Add 40 μl of probe/formamide mix to Eppendorf tubes containing hybridization solution and slowly mix by pipetting up and down 15×.
3. Apply 160 μl of probe/hybridization mixture evenly to the slide by carefully pipetting along the section (*see* **Note 26**).
4. Carefully cover slides with HybriSlip covers without making bubbles. Use one pair of forceps to hold the HybriSlip in place while slowly lowering it onto the slide with another pair of forceps.
5. After applying the hybridization solution, immediately place the slides in a prewarmed box humidified with 50% formamide (standard grade). Incubate overnight at the optimal hybridization temperature (*see* **Note 27**).

3.5 Washing and Antibody Reaction (Day 6)

Preheat two 500 ml beakers containing 200 ml 0.2× SSC solution to 55 °C in an incubator.

3.5.1 Coverslip Removal and High Stringency Washes

1. Fill two 500 ml beakers with 300 ml 2× SSC. Individually remove each slide from the hybridization box with forceps, and dip up and down in the first beaker of 2× SSC until the coverslip falls off (*see* **Note 28**).
2. Dip each slide up and down a few times in the second beaker to rinse off excess hybridization buffer, and place in a staining dish containing 2× SSC. Repeat this and the above step for each slide.
3. After each slide has been processed, transfer them to a second staining dish with fresh 2× SSC and incubate at room temperature for 5 min.
4. Transfer slides into the first 500 ml beaker containing prewarmed 0.2× SSC and incubate for 1 h at 55 °C. Dip slide rack up and down a few times during the incubation. Alternatively, use a water bath with a shaking platform.
5. Transfer the slides to the second beaker with 0.2× SSC and incubate for another 1 h at 55 °C dipping up and down a few times.

3.5.2 Blocking and Antibody Reaction

1. Remove slide holder from beaker and place it into a staining dish containing 1× MN buffer. Dip slides up and down 15× and then incubate at room temperature for 10 min.

2. Transfer each individual slide into a shallow plastic tray filled with 1% blocking reagent solution. Incubate at room temperature for 30 min with gentle rotation on a rotating platform. Repeat 1× (*see* **Note 29**).
3. Transfer each individual slide into a shallow plastic tray containing 1% BSA solution. Incubate at room temperature for 30 min with gentle rotation. Repeat 1×.
4. Dilute required amount of antibody in 1% BSA solution (*see* **Note 30**).
5. Fill slide mailers with 16 ml of antibody/BSA solution. Transfer each individual slide from 1% BSA solution to a slide mailer (5 slides/mailer).
6. Incubate at room temperature for 2 h with slow rotation.
7. Transfer slides to a plastic tray containing 1% BSA solution and incubate at room temperature for 20 min with slow rotation. Repeat 3× (*see* **Note 31**).

3.6 Colorimetric Reaction and Mounting (Days 6–7)

3.6.1 Colorimetric Reaction

1. Dip slides up and down 15× in TNM5 buffer and incubate for 2 min. Repeat 2×.
2. In 50 ml conical tube, dilute 500 µl NBT/BCIP Stock Solution in 50 ml TNP buffer. Add 16 ml of this solution to each slide mailer (5 slides/mailer) and carefully place slides into mailers making sure that the sections are completely submerged (*see* **Note 32**).
3. Incubate at room temperature in complete darkness with no movement (*see* **Note 33**).

3.6.2 Mounting

1. When the color reaction is complete, wash the slides in 1× TE buffer for 5 min. Repeat 2×.
2. Place slides on a clean sheet of aluminum foil with sections facing up. Apply 2–3 drops of Aqua-Poly/Mount and cover with a glass coverslip. Gently squeeze out the air bubbles and excess mounting medium by pressing down gently on the coverslip with a folded Kimwipes.
3. Let slides dry overnight at room temperature to harden the mounting medium.
4. Clean slides and examine under the microscope.

4 Notes

1. DEPC should not be used to treat solutions with high concentrations of Tris such as TE and proteinase K buffer.

2. When adjusting pH for RNase-free solutions clean the glass electrode with RNaseZap and rinse with DEPC-treated water.
3. All solutions up to this point must be made from RNase-free components.
4. Use NaOH pellets during pH adjustment.
5. See https://www.qiagen.com for details and useful tools for calculating RNA melting temperatures and self-complementarities, but we recommend the following general principles during LNA probe design. LNA bases have significant differences in binding strengths (C > T > G ≫ A). Try to design probes with low self-complementarity, 30–60% GC content, avoid ≥4 sequential LNAs, avoid ≥3 sequential LNA-modified G's or C's and do not place LNA-modified bases at oligo termini.
6. Be careful not to open siliques up too much in order to not lose ovules. We generate a perforated cut to keep valves attached but allow them to be subsequently removed easily.
7. Collection and fixation of siliques must be carried out on ice.
8. If siliques are floating in ethanol wash series (Subheading 3.2, **steps 2** and **3**), then vacuum infiltrate (described in Subheading 3.2, **step 1**) until siliques sink to the bottom of scintillation vials.
9. Eosin Y allows better visualization of tissues during embedding and sectioning.
10. At this point siliques can be stored for up to a few weeks at 4 °C in 70% ethanol. First, wash siliques with 85% and 70% ethanol for 5 min each.
11. Paraffin embedding can also be done by hand in case an automatic embedding machine is not available. First, tissues need to be cleared with either xylenes or HistoClear as the clearing reagent. Remove 100% ethanol from previous step and replace with 25% clearing reagent/75% ethanol in glass scintillation vials. Incubate for 30 min and repeat with 50% clearing reagent/50% ethanol, 75% clearing reagent/25% ethanol and 100% clearing reagent. Repeat step with 100% clearing reagent. Place a beaker containing paraplast chips in an incubator and let melt for >6 h. Add 20 chips (approximately 2 g) to glass scintillation vials, place in incubator set to 42 °C and swirl occasionally until paraplast is melted. Repeat this until vials are full (4–5×) and increase temperature as needed to fully melt paraplast. Remove clearing reagent from vials containing fixed/cleared tissues and add melted paraplast. Mix by swirling vials and incubate at 60 °C for ≥4 h. Exchange melted paraplast each morning and night for 2 days (4× total).
12. Place paraffin blocks on ice for ≥30 min before sectioning.

13. Use paintbrushes or cotton-tipped applicators to handle ribbons and orient sections on slides.
14. Store solutions and buffers for in situ hybridizations on a designated RNase-free shelf/bench area separate from other lab chemicals.
15. Paraffin blocks can be stored for weeks at 4 °C but after sectioning try to process the slides as soon as possible.
16. Originally xylenes were used to clear tissues and remove paraffin from tissue sections. However xylenes are toxic and Histo-Clear is a suitable replacement.
17. If the sections are still stained with eosin Y after the 85% ethanol wash, then let sections incubate an extra 5 min in 85% ethanol until the dye is not visible.
18. Keep the ethanol series (except 100%) in separate staining dishes to use again during the dehydration step.
19. Prewarm staining dish containing proteinase K buffer to 37 °C before this step.
20. Proteinase K is used to partially digest the tissue to allow better probe penetration. However, over digestion reduces specimen integrity. Therefore, the ideal duration of proteinase K incubation needs to be carefully calibrated for each tissue type. These conditions were optimized for digesting sectioned embryos embedded within siliques.
21. Use the alcohol solutions from the hydration steps, except for the 100% ethanol because it is contaminated with clearing reagent.
22. Alternatively, a layer of Parafilm can be used to prevent direct contact between slides and paper towels soaked with formamide.
23. Dextran sulfate is viscous and very difficult to pipet. It helps to prewarm aliquots to room temperature before pipetting.
24. Hybridization solution should be made fresh for each set of hybridizations.
25. The optimal probe concentration should be determined for each probe individually. To reduce background, use the lowest possible probe concentration. For a new probe try different concentrations ranging from 5 nM to 100 nM. In our experience, 20 nM final concentration typically gives optimal results for early embryos. Prepare 1 mM working solutions from LNA probe stock (100 mM) and store at −20 °C.
26. The amount of hybridization solution depends on the number of sections on the slide, and on the density and thickness of the sections. Based on our experience, 160 μl/slide works well.

27. The optimal hybridization temperature needs to be experimentally determined. In our experience, 65 °C works well for probes designed according to **Note 5**.
28. Do not remove coverslips manually with forceps and be careful not to damage the sections during this step.
29. Be careful not to rotate too fast. This can cause the slides to slide over each other and damage the specimen.
30. The optimal antibody dilution may change with different probes; however we typically use a 1:1500 dilution.
31. Add slides to mailers slowly and make sure all slides are covered in the color reagent evenly. Do not use slide mailers that were used for the antibody binding step.
32. Because TNP buffer is viscous, pour into conical tube and then pipet in the NBT/BCIP solution. Close screw cap and shake vigorously to mix. Cover with aluminum foil and protect from light.
33. For a typical probe, monitor the colorimetric reaction after ≥15 h. If you expect a very strong signal, begin monitoring after only a few hours. Choose a test slide and rinse with TNM5. Wipe off the back of the slide and observe using an old microscope. Put the test slide back into the color solution if the signal is weak.

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