The in situ hybridization protocol outlined below is an excerpt from the following chapter to be published in *Methods in Molecular Biology*:

In situ hybridisation as a tool to study the role of miRNAs in plant development.
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2. Materials

All solutions are prepared using RNase-free glassware and chemicals in DEPC-treated dH2O (water with DEPC 0.05% is stirred overnight at room temperature, and subsequently autoclaved).

2.1. Tissue preparation

2.1.1. Fixation

4% Paraformaldehyde (Sigma P6148)
Fixative must be prepared fresh on the day of use. Make up the required amount of phosphate buffered saline (PBS): 130 mM NaCL, 7 mM Na2HPO4, 3 mM NaH2PO4, in DEPC-treated water. Adjust the pH to 11.0 with NaOH. The pH can be checked with pH papers and disposable RNase-free plastic pipettes. Heat the solution to 60-70°C. In a fume hood, add paraformaldehyde (4%) and mix thoroughly until dissolved. Place the solution on ice and when cooled adjust the pH to 7.0 with H2SO4 (1-2 drops for 100ml). Do not use HCl to adjust the pH as this will release highly toxic fumes. To improve the infiltration of the fixative, the following detergents can be added:
DMSO to 4%, Triton to 0.1%, or Tween to 0.1 - 0.3%.

2.1.2. Embedding

1. PBS
2. 100% EtOH

3. 8.5% NaCl 1 litre
4. Histoclear Fisher
5. Tissuepath paraplast X-tra Fisher 23-021-401
   Paraplast should be freshly melted before use in an oven at 58-60°C. However, prolonged heating above 60°C should be avoided.
6. Base moulds EMS 62352-15
7. Embedding rings Fisher 22-038-197

2.1.3. Sectioning

1. Probe-on-plus slides Fisher 15-188-52
2. 0.2N NaOH

2.2. Probe preparation

2.2.2. In vitro transcription

1. Phenol/chloroform
2. DIG RNA labelling kit Roche 1-175-025
3. RNasin
4. RNA polymerase
   Transcripts are made from linearized plasmid templates that typically carry promoters for one of the following RNA polymerases T3, T7, or Sp6.

2.2.3. DNase treatment

1. RNase-free DNase (RQ1) Promega M610A
2. 100 mg/ml tRNA (store at -20°C) Roche 109541
3. 4M NH4Ac 100 ml
4. 100% EtOH

2.2.4. Carbonate hydrolysis

1. 2x CO3-buffer 50 ml

80 mM NaHCO3

120 mM Na2CO3

2. 10% Acetic acid 50 ml
3. 3M NaAc pH 5.2 100 ml
4. 50% deionized formamide (store at 4°C) Sigma F9037

2.3. in situ hybridization

The following general stock solutions should be prepared. Again, all solutions are prepared using RNase-free chemicals and DEPC-treated dH2O. In addition, approximately 12 L DEPC-treated dH2O is needed in 1L RNase-free bottles for diluting stock solutions.

1. 10X PBS pH 7 1 litre

   1.3 M NaCl

   70 mM Na2HPO4

   30 mM NaH2PO4

   This solution should come out to pH 7, but if necessary the pH can be adjusted with H3PO4 or NaOH. Check the pH with pH paper.
2. 0.5M EDTA pH 8 500ml

   adjust the pH with NaOH
3. 1M Tris solutions at pH 9.5, pH 8, and pH 7.5 1 litre

   Adjust the pH with concentrated HCl.

   Tris contains an amino group, which inactivates DEPC so it is best made up with Tris powder from a dedicated clean stock and dissolved in DEPC treated water.
4. 5M NaCl 500 ml
5. 100mM NaPO4 buffer pH 6.8

   For 200ml mix 51ml 200mM NaH2PO4, 49ml 200mM Na2HPO4, and 100ml dH2O
6. 1M MgCl2 500 ml

2.3.1. Section pretreatment

1. Histoclear
2. 100% ethanol
3. 20X SSC 1 litre

3 M NaCl

300 mM Na Citrate

4. 10 mg/ml Proteinase K

   Proteinase K does not need to be predigested. Aliquots of this stock solution are stored at -20°C. For maize tissues, we frequently use Pronase instead of Proteinase K.

   40 mg/ml Pronase (Sigma type XIV) is dissolved in water and pre-digested to remove nucleases by incubating at 37°C for 4 hours. Aliquots are stored at -20°C.

5. 20% Glycine in 10x PBS (store at -20°C)
6. 4% Paraformaldehyde in PBS, made fresh as detailed above in 2.1.1.
7. Acetic anhydride 4 ml in 800 ml 0.1 M triethanolamine-HCl pH 8.

   Acetic anhydride is unstable in water thus this solution should be made fresh immediately befor use. Place a RNase-free glass container over a magnetic stirrer in the fume hood and add the following solutions while stirring vigorously: 786 ml DEPC dH20, 10.4 ml triethanolamine (Sigma T1377), 3.2 ml HCl, when the solution is mixed add 4 ml acetic anhydrite (Sigma A6404). Lower the mixing speed and immediately lower the slide rack in the solution. Whereas the other solutions can be used for multiple slide racks, this solution should be made fresh for each rack of slides.

2.3.2. Hybridisation

1. 10X in situ salts 50 ml

   3 M NaCl

   100 mM Tris pH 8

   100 mM Na Phosphate pH 6.8

   50 mM EDTA

2. 100 mg/ml tRNA (store at -20°C) Roche 109541
3. 50% Dextran Sulfate (MW > 500,000) 40 ml (store at -20°C) Sigma D8906-5G

   Dextran Sulfate should be heated to 80°C to dissolve.

4. 50X Denhardts (store at -20°C) Sigma D2532
5. formamide (store at 4°C) Sigma F9037

2.3.3. Post-hybridization treatment

1. 20X SSC 1 litre

3 M NaCl

300 mM Na Citrate

2. 5X NTE 1 litre

   2.5 M NaCl

   50 mM Tris pH 8

   5 mM EDTA

3. 20 mg/ml RNase-A (store at -20°C) Roche 109142

2.3.4.Detection of the hybridization signal

1. TBS 1litre

   100 mM Tris pH 7.5

   150 mM NaCL

2. 1% Blocking reagent (Roche 1 096 176) in TBS 200 ml

   TBS should be heated to 60°C, Blocking reagent should be added on top of the solution and stirred for at least 1 hour to dissolve completely.

3. 1% BSA (Sigma A7906), 0.3% Triton X-100 in TBS 500 ml
4. Anti-DIG-Alkaline Phosphatase, Fab fragments Roche 1 093 274
5. TN 250 ml

   100 mM Tris pH 9.5

   100 mM NaCl

6. NBT/BCIP mix (store at -20°C) Roche 1 681 451

   Just before use, add 200 ul NBT/BCIP mix to 10 ml TN. Alternatively a premix of NBT/BCIP called "Western Blue" (Promega S3841) can be used.

7. TE pH 8 500 ml

   10 mM Tris

   1 mM EDTA

8. 100% EtOH
9. Histoclear
10. Cytoseal EMS 18006

Equipment

Vacuum system

Fume hood

Oven at 58-60°C to melt the paraplast

Ovens for 50°C, 55°C and 37°C (need two due to the quick change between temperatures)

Slide warmer

Microtome

Metal slide rack to fit in glass trough

Three glass troughs baked at 250°C overnight

Stir bars, spatulas and glass measuring cylinders baked at 250°C overnight

21 plastic boxes, soaked overnight in 0.2M NaOH and rinsed in DEPC-treated water.

Two large flat plastic boxes with sealable lids for hybridisation and antibody treatment

Plastic disposable pipettes, individually wrapped

Disposable plastic Pasteur pipettes

3. Methods

3.1. Tissue preparation

Before you start, use northern blots or RT-PCR to assess the expression levels of the gene of interest in the various tissues of the plant to ensure that appropriate tissues or developmental stages are analyzed.

3.1.1. Fixation

1. Harvest samples and place as quickly as possible in fresh fixative on ice, either in 15ml blue falcon tubes or glass scintillation vials. If dissection is required, this is best done on ice in cold fixative. For instance, maize apices are dissected into small 4x4x3 mm blocks to optimize the infiltration of fixative and subsequent solutions.

2. Apply vacuum (~400 mm Hg) to samples while on ice. Small bubbles should release from the samples. Hold vacuum for 15-20 minutes and release slowly.
   Ensure that the fixative does not boil. Repeat this step until tissues sink. Tissue samples that are more difficult to infiltrate can be placed in special tissue holders such that the tissue is fully submerged in fixative.

3. Replace the paraformaldehyde with fresh fixative and gently shake overnight at 4°C. Use a large excess of each solution, e.g. for 8-10 maize apices or Arabidopsis seedlings use 10-15 ml.

3.1.2. Embedding

Machines for automatic wax embedding are available and can be used to simplify this step.

1. Pre-cool the following solutions by keeping them in the cold room.
   All steps are done at 4°C with gentle shaking or rotation:

1X PBS	30 min
1X PBS	30 min
30% EtOH	60 min
40% EtOH	60 min
50% EtOH	60 min
60% EtOH	60 min
70% EtOH	60 min
85% EtOH	60 min
95% EtOH	overnight.

   Addition of 0.1% eosin to the final step helps to visualize small samples when sectioning.
   0.85% NaCl can be added to the 30-85% EtOH solutions to avoid excessive swelling and shrinking of the tissue.
   Tissue can be stored for several months in 70% EtOH at 4°C.

2. If the initial steps are performed in 15ml blue falcon tubes, the samples should be transferred to glass vials before the histoclear is added as static can otherwise be a problem.
   All steps are done at room temperature with gentle shaking:

100% EtOH + eosin	30 min
100% EtOH + eosin	30 min
100% EtOH + eosin	60 min
100% EtOH + eosin	60 min
25% histoclear, 75% EtOH	30 min
50% histoclear, 50% EtOH	30 min
75% histoclear, 25% EtOH	30 min

100% histoclear	60 min
100% histoclear	60 min
100% histoclear + 1/4 volume paraplast chips	overnight

3. Place the samples at 42°C until chips are completely melted (this may take several hours).

4. Add more chips and move to 60°C.
   At the end of the day, replace the wax/histoclear with freshly melted wax and leave the vials open overnight at 60°C
   An RNase-free beaker of wax should be set up and replenished at each change so there is always freshly melted wax at hand.

5. Change the wax each morning and evening of days 5 and 6.
   Leave the vials open at 60°C overnight.

6. In the morning of day 7, replace the wax once more.
   Samples can be embedded later that day.
   Pour some paraplast into a mould and using warmed forceps arrange the tissue sample in the mould. Place the moulds on ice to aid quick setting.
   We either orientate plant tissues carefully in the moulds to fit onto the microtome and be sectioned directly. Alternatively, we embed samples randomly and in bulk in brand-new pie dishes (aluminium tins about 15 cm in diameter). Individual samples can later be cut out and using more hot wax be mounted in the correct orientation onto microtome blocks.

   If wax infiltration appears to be a problem, as evidenced by holes in the tissue sections, samples can be vacuum infiltrated once more immediately prior to making the blocks. However, make sure that the vacuum system is warmed up to 58-60°C
   Special hotplates are available that maintain 60°C and simplify the embedding process.
   Blocks can be stored in plastic bags at 4°C for one year or longer.

3.1.3. Sectioning

Sectioning can be tricky in very dry conditions, when static becomes a problem, or in warmer temperatures, when the ribbon may buckle in the heat. The wax blocks can be sectioned from cold and

sometimes ‘earthing ‘ the microtome with a wire helps reduce static. Slides used are Probe-On Plus from Fisher Biotechnology. They are pre-cleaned and charged. They also have a white paint label on them that makes a capillary space when you sandwich two together.

1. Pre-warm the slide warmer to 42°C.
   Wipe over microtome and slide warmer with 0.2M NaOH to clean.
   Lay down clean filter paper next to microtome to place wax ribbons on.

2. Trim the block into a trapezoid shape leaving about 2 mm wax around the plant tissue. Place the block into the microtome such that the longer of the two parallel faces is at the bottom. Section through the region of interest. Sections 8 - 10µm thick are reasonable depending on the size of cells in your tissue. The sections should make long wax ‘ribbons’.

3. Place a Probe-On Plus slide on the slide warmer and apply several drops of DEPC treated water. Slides can be marked with pencil, as this will not dissolve in later EtOH incubations.

4. Float wax ribbon on the water, ‘shiny’ side down (the under side as the ribbon comes off the microtome). Let the ribbon warm for approximately a minute to allow it to flatten out completely.

5. Before the edges of the wax contact the warm slide tip off the water carefully but in one smooth movement, so the ribbon is lowered down onto the slide. Hold the slide upright and use a twist of tissue to drain off excess water from the edge of the ribbon.

6. Leave the slides under a slight angle on the slide warmer at 42°C overnight so that the tissue adheres. Sectioned tissue can be stored in a box with silica desiccant for several weeks at 4°C.

3.2. Probe preparation

3.2.1. Designing the probe

Most routinely, PCR products derived from cDNA or genomic DNA are cloned into the pCRII-TOPO vector (invitrogen). This vector includes an Sp6 and T7 promoters allowing in vitro transcription in either direction across the insert. Several other vectors are available that contain T7, T3, or Sp6 promoters.

3.2.2. In vitro transcription

1. Linearize the plasmid by digesting approximately 5 ug DNA with a restriction enzyme that digests at the end of the insert opposite the site of the promoter.
   Do not use restriction enzymes that leave a 3' overhang. This can lead to transcription artefacts because the polymerase can utilize the 3' overhang as a substrate to continue transcription. Digest for three hours and use extra enzyme to be sure of a complete digest.

2. Check an aliquot of the digest on a gel to verify that the reaction has gone to completion.

3. Extract the DNA with phenol/chloroform, precipitate with EtOH and resuspend in DEPC-treated dH2O to a concentration of 0.5ug/ul. Alternatively, the digest can be cleaned using standard DNA purification columns.

4. Set up the transcription reaction as follows:

DNA (0.5ug/ul)	4ul
5x buffer	5ul
5x nucleotides	5ul
RNase inhibitor	to 1unit/ul
RNA polymerase	to 0.4 units/ul
H20	to 25ul

   5X nucleotides are 2.5mM ATP, GTP, CTP, UTP/DIG-UTP in H2O.

   Incubate at 37°C for 30 - 60 minutes.

   We use a 1:1 DIG-UTP : regular UTP ratio because this gives a much better RNA yield.
   We have experienced that longer incubations times up to 2 hours can improve the RNA yield for certain miRNA probes.

5. Treat a gel box and comb with RNase zap (Sigma) or 0.2N NaOH for 30 minutes. Make up a fresh 1% gel that contains ethidium bromide. Run 1 ul of the transcription reaction at 80V for

about 15 minutes. For a good in vitro transcription reaction, the RNA band will be at least as intense in brightness as the DNA band. Use a control RNA, which is usually provided with the in vitro transcription kit, to estimate the amount of probe synthesized. Alternatively, the relative intensity of the RNA and DNA bands can be used to estimate the probe yield. Depending on the concentration at which the probe will be used (which will vary for different genes) 2ug of probe is usually sufficient for 80 slides.

3.2.3. DNase treatment

To remove the DNA template from the in situ hybridization probe, the in vitro transcription reaction is treated with DNaseI.

1. To the transcription reaction add:

DEPC-H20	75 ul
tRNA (100mg/ml)	1 ul
RNase-free DNase (RQ1)	5units

   Incubate for 10 minutes at 37°C

   The tRNA serves as a carrier RNA in the subsequent precipitation reactions.

2. Add an equal volume of 4M NH4Ac plus 2 volumes of EtOH, and precipitate the RNA at -20°C for an hour
   Spin down the RNA pellet at 4°C for 30 minutes
   Rinse the pellet with 70% EtOH and let it air dry for 15 – 30 minutes on ice.

3. Resuspend the pellet in 100 ul DEPC-H2O

3.2.4. Carbonate hydrolysis

The probe is usually partially hydrolysed to yield fragments of about 150 nt. Shorter probes can better penetrate the tissue and consequently improve the hybridization signal and reduce the background. As some of our miRNA probes are about this length, we sometimes skip this step.

The length of time required to hydrolyze probes of different sizes is calculated using the following formula:

Time = Li - Lf / K Li Lf
Li = initial length of probe (in kb)
Lf = final length of probe (0.150 kb)
K = 0.11 kb/minute

1. Add 100 ul 2x CO3 buffer (80 mM NaHCO3, 120 mM Na2CO3) and incubate at 60°C for the calculated length of time.

2. Neutralize the reaction with 10ul 10% acetic acid

3. Add 1/10 volume 3M NaAc pH 5.2 plus 2 volumes EtOH and precipitate the RNA at -20°C for an hour.
   Spin down the RNA pellet at 4°C for 30 minutes
   Rinse the pellet with 70% EtOH and let it air dry for 15 – 30 minutes on ice.

4. Resuspend the RNA in 50% formamide at a final concentration of 50 ng/kb/ul.

Most probes are used at a final concentration of 0.5 ng/kb/ul probe complexity, so this gives a 100x stock for making up the hybridization solution. Hybridizations are done in 100 ul per slide. Thus, if a probe is 0.5 kb long the probe should be resuspended at a concentration of 25 ng/ul (50 ng x 1 ul x 0.5 kb) to obtain a 100x stock. If the probe is 1 kb long, 50 ng/ul probe will make a 100x stock.

Probes can be stored for months at –80°C, allowing you to use the same probe as a control in different experiments.

3.3. in situ hybridization

3.3.1. Section pre-treatment

Before the hybridization, sections are pretreated to optimize the signal intensity and specificity. Tissue sections are first dewaxed and rehydrated through an ethanol series. The sections are subsequently treated

with Proteinase K, which increases the permeability of the tissues, thus enhancing the infiltration with the RNA probe. Some experimentation may be needed to determine the optimum conditions, time and temperature, to maximize the hybridization signal without breakdown of the tissue. Proteinase K activity is stopped with glycine. The sections are fixed again with paraformaldehyde, and then treated with acetic anhydride to neutralise positive charges that may lead to non-specific binding of the probe.

The volume required for each solution will obviously depend on the number of slides to be treated and the container size used. The sections should always be completely submerged. For a 50-slide rack and a neatly fitting container, 600 ml should be sufficient. Because many of the incubation steps are very short we usually prepare all possible solutions, including the 4% paraformaldehyde (see section 2.1.1), first and then start the slide pre-treatments. However, the acetic anhydride solution will need to be prepared immediately before use and proteinase K is added at the time of use.

All steps are performed at room temperature unless noted otherwise.

1. Select the slides and label each in pencil with the probe to be used. Be sure to pick pairs for each probe as the hybridisation steps are done with 'sandwiched' slides.

2. Warm 600ml of 100 mM Tris pH 8, 50 mM EDTA to 37°C.
   (60ml 1M Tris pH8, 60ml 0.5M EDTA for 600ml)
   Add 60ul 10mg/ml proteinase K just before step 3.

3. Deparaffinize and rehydrate the tissue sections:

histoclear	10 min (use a glass dish)
histoclear	10 min (use a glass dish)
100% EtOH	1 min
100% EtOH	1 min
95% EtOH	1 min
90% EtOH	1 min
80% EtOH	1 min
60% EtOH	1 min
30% EtOH	1 min
H2O	1 min

4. 2X SSC 15-20 min

5. 100 mM Tris 8, 50 mM EDTA with freshly added proteinase K (1µg/ml) 30 min at 37°C

6. 0.2% glycine in PBS 2 min

7. PBS 2 min

8. PBS 2 min

9. 4 % paraformaldehyde pH 7 (made fresh) 10 min

10. PBS 5 min

11. PBS 5 min

12. acetic anhydride 10 min

While the slides are in the PBS washes, make up 0.1 M triethanolamine buffer pH 8 in a glass dish (see section 2.3.1) by mixing on a magnetic stirrer in the fume hood:

DEPC H2O	786.4ml
Triethanolamine	10.4ml
Conc. HCl	3.2ml

Add 4 ml acetic anhydride into the triethanolamine immediately before putting the slides in, and stir well. Reduce the speed of the stirrer and elevate the slide rack in the container of triethanolamine / acetic anhydride (we use an second inverted slide rack to support the samples). After adding the slides, continue to stir slowly for 10 minutes.

13. PBS 5 min

14. PBS 5 min

15. Dehydrate again:

30% EtOH	30 sec
60% EtOH	30 sec
80% EtOH	30 sec
90% EtOH	30 sec
95% EtOH	30 sec
100% EtOH	30 sec
100% EtOH	30 sec

16. Slides can be stored in container with a small amount of 100% EtOH at the bottom for up to several hours at 4°C.

Both paraformaldehyde and acetic anhydride are toxic, so these solutions should be prepared in a fume hood and properly disposed off.

3.3.2. Hybridisation

We do not usually include a sense "control" probe, as this RNA is entirely different in sequence and not a true negative control. However, if a RNA null mutant exists, such tissues would be an excellent negative control. We always include a positive control, most often *SHOOTMERISTEMLESS* or *knotted1*, which are relatively abundant. As mentioned earlier, most probes are used at a probe complexity of 0.5 ng/kb/ul. However, for new probes or new tissues, a dilution series of the probe should be tried. We often try 1x, 5x and 20x that concentration.

1. Decide what slides to use with which probe. Determine how much hybridization solution to make based on the total number of slide pairs.

   Hybridization Solution (enough for 5 slide pairs)

10X in situ salts	100 µl
deionized formamide	400 µl
50% dextran sulfate	200 µl
50X denhardts solution	20 µl
tRNA (100 mg/ml)	10 µl
DEPC-H20	70 µl

total volume: 800 µl

This solution is very viscous from the dextran sulfate. Either warm it up before use, or make more than you need to overcome loss in dispensing. Hybridization solution can be made up in bulk and stored in aliquots at –20°C.

2. Air dry the slides by leaving the slide rack covered with a kimwipe in a clean dry box whilst you prepare the solutions. The slides must be completely dry.

3. For each pair of slides, probe should be added to 50% formamide such that the total volume is 40 µl. Heat to 80°C for 2 min, immediately chill on ice, spin down briefly and keep on ice.

4. Add 160 µl of hybridization solution for each pair of slides such that the volume is now 200µl (hybridization solution + probe) for each slide pair (see table below for mixing up volumes of hybridization solution). Mix slowly and carefully to avoid bubbles.

Hybridsation solutions table

Pairs of slides	Probe(µl)	50% formamide(µl)		Hyb solution(µl)	Final vol. (µl)	
1	2	38		160	200	Apply 200µl to each pair of slides
2	4	76	80°C 2min	320	400	
3	6	114		480	600	
4	8	152		640	800	
5	10	190		720	1000	
*1	10	30		160	200	

This assumes you have the probe at 50ng/kb/ul.
*5X probe.

5. Apply the probe to the slides. Because the sections are dried and the probe is viscous, care should be taken to infiltrate all sections and avoid air bubbles. We use one of three methods:
a. Excess amounts of the hybridisation solution are put in a plastic trough designed for multiwell pipettors (Matrix Technologies). Two slides are sandwiched together and the slide sandwich is dripped into the tough on its side. This allows the solution to be pulled up by capillary action.

b. A second technique is to apply 200 ul probe to the long edge of one slide, then make a sandwich by gradually lowering the second slide down. Tap on the slides if the probe seems to exclude a section.

c. A third technique is to apply 100 ul probe to each slide, carefully spreading it out over the entire slide with the side of a pipette tip so that all sections are covered. Then slowly sandwich the two slides together without getting bubbles. If bubbles do arise, do not pull the slide apart but tap the slides to displace the bubbles from all tissue sections, add additional hybridization solution if needed.

6. Line a plastic box with damp tissue paper and use plastic pipettes to make racks for the pairs of slides. The slide sandwiches can be stacked on top of each other, but they should not touch on the side as this will lead to mixing of the probes. Seal the box tightly.

7. Hybridize at 50°C overnight.

3.3.3. Post-hybridization treatment

The slides are washed of the hybridisation solution and treated with RNase A to remove single stranded RNA and to increase the specificity of the probe. RNase A will cleave at mismatches in RNA : RNA hybrids. As a result, probe that cross-hybridizes with potential closely related sequences will be fragmented and washed off in the subsequent washes.

Warm 0.2X SSC to 55°C (need ~3L)
Warm NTE solution to 37°C (need ~3L)
These solutions are usually prepared the evening before and warmed up overnight.

During one of the wash steps, the 200 ml blocking solution should be prepared (see section 2.3.4.):

1% Blocking (Roche) reagent in 200 ml TBS.
TBS should be heated to 60°C. Blocking reagent should be added on top of the solution and stirred for at least 1 hour to dissolve completely. The solution will stay cloudy. Do not heat over 60°C.

All washes are done with prewarmed solutions and with gentle shaking

1. Dip pairs of slides into a dish of prewarmed 0.2x SSC to separate and rinse them before placing in a rack.

2. Wash in 0.2x SSC at 55°C for 60 min

3. Replace the 0.2x SSC and wash at 55°C for 60 min

4. Wash in NTE at 37°C for 5 min

5. Replace the NTE and wash at 37°C for 5 min

6. Incubate with RNase (20 μg/ml) in NTE with gentle shaking at 37°C for 30 min
   Add RNase to prewarmed NTE immediately before use

7. Wash in NTE at 37°C for 5 min

8. Replace the NTE and wash at 37°C for 5 min

9. Wash in 0.2x SSC at 55°C for 60 min

10. PBS at room temp for 5 min

3.3.4. Detection of the hybridization signal

The slides are first blocked to prevent non-specific cross-reaction with the anti-DIG antibody.

1. Place slides on the bottom of a large flat plastic container, tissue side up. Add 1.0% Roche block (see above); use just enough Blocking solution to cover the slides. Place the container on a rocking platform, rock gently and incubate at room temperature for 45 min.

At this time, make up 500 ml 1% BSA, 0.3% Triton X-100 in TBS (see section 2.3.4):

BSA	5 g
1M Tris pH 7.5	50 ml

5M NaCl	15 ml
Triton-X 100	1.5 ml

2. Replace blocking solution with 1% BSA, 0.3% Triton X-100 in TBS, and wash for 45 min as in step 1.

3. Dilute anti-DIG antibody (1:1250) in 1% BSA, 0.3% Triton X-100 in TBS solution. 10µl antibody in 12.5ml is sufficient for 25 pairs of slides.

4. Put a few ml of antibody solution in a small dish. We often use plastic multipipetting or weighing dishes. Sandwich slides together and dip one long side in the solution, allowing capillary action to pull up the solution. Drain on Kimwipe and fill up again. You may need to tap the slides as the solution flows in to avoid bubbles.

5. Arrange slides on racks of plastic pipettes above wet tissue in a tightly sealed plastic container and leave overnight at 4°C.

6. Drain slides on Kimwipes and separate. Place them on the bottom of a plastic container as in step 1. Wash 4x in 1% BSA, 0.3% Triton X-100 in TBS solution on a rocking platform at room temperature for 15 min each.

7. Wash in TN (100 mM Tris 9.5, 100 mM NaCl) for 10 min

8. Dip each slide in TN solution to ensure that all detergent is washed off and the pH in the sections is raised to 9.5 for optimum alkaline phosphatase activity.

9. Prepare substrate solution immediately before use by adding 200µl Pre-mixed NBT/BCIP (Roche 1 681 451) to 10 ml TN solution. This is enough for 25 slide pairs.

10. Sandwich two slides and draw up the substrate solution as in step 4. Repeat.

11. Place slides in plastic container above wet paper towels in total darkness for 1-5 days. Check development on a dissection microscope with the slide sandwich balanced on an opened Petri dish to avoid that capillary forces drain the substrate. Replace the substrate solution every other

day by pulling out old solution onto Kimwipes and bringing up fresh substrate via capillary action as in step 4.

12. When the signal is clear, drain the slide pairs, separate the slides, and rinse them in TE to stop the alkaline phosphatase reaction.

13. Dehydrate the sections through an EtOH series:

30% EtOH	5 sec
50% EtOH	5 sec
70% EtOH	5 sec
85% EtOH	5 sec
95% EtOH	5 sec
100% EtOH	5 sec
histoclear	2 min
histoclear	2 min

It is important to keep the time in EtOH to a minimum because the color product is alcohol soluble.

14. Dry the slides in a fume hood and mount them with a few drops of Cytoseal. Leave the slides flat overnight to dry.

The EtOH series can be skipped. Instead, rinse the slides in milli-Q water and dry them at 37°C in a box with Kimwipes to ensure thorough dryness. Then proceed with the mounting in cytoseal as above.

4. Notes

Some prefer to use pronase instead of proteinase K to permeabilize the tissue sections. To do so, the pre-treatment steps following the 1 min incubation in H2O, steps 3.3.1.4 and 5, can be modified as follows:

4.	PBS	5 min
5.	pronase	10 min

Pre-digested pronase (see section 2.3.1) is diluted to a final concentration of 0.125 mg/ml in 50 mM Tris pH 7.5, 5 mM EDTA

Because of the number of solutions used and the length of the protocol, it is important to have everything to hand before you start to save a panicky run about making up solutions during a 5 minute wash. The timetable below is what we use, though the protocol can be worked through in two long days by reducing the washes in 3.3.3 to 30 min and the antibody incubation step to 4 hours at room temperature.

A dedicated in situ space is very useful and lessens the worry about RNases 'floating in' from adjacent experiments. You also need a whole clean lab bench to lay out all the boxes of solutions during the de- and re- hydration steps, and quick access to a fume hood for the histoclear, acetic anhydride and paraformaldehyde steps.

It is usual to make all solutions RNase-free by DEPC treatment. However, if you have good clean milliQ water this may not be necessary and DEPC does interfere to some extent with transcription reactions and is a potent carcinogen so best to do without it if possible. We use falcon tubes to measure most RNase-free solutions.

Treat plastic containers with 0.2M NaOH overnight and rinse with DEPC water. They can be kept from experiment to experiment as long as they are not stored for more than a week or so. We keep boxes that are used before the RNase step separated from those boxes used for RNase treatment or thereafter.

Timetable

Day 0

Prepare the probes
Prepare and label slides
Made up stock solutions
Prepare 21 RNase free boxes
Bake slide rack, stir bars, three glass boxes and one 500ml glass measuring cylinder

Day 1

Preparation before lunch:
Make up EtOH series and histoclear
Make up 2x SSC, PBS, PBS/glycine
Warm Proteinase-K buffer

Make up Paraformaldehyde

12-3 pm
Pretreat the slides as outlined in 3.3.1

4 pm
Aliquot the probes and hybridization solutions, see 3.3.2
Add probes to the slides (about 30min for 10 slide pairs)
Incubate slides at 50°C overnight.

Make up 3 litres each of NTE (37°C) and 0.2x SSC (55°C) and set to warm.

Day 2
Before noon:
Start the post-hybridization washes, see 3.3.3
Make up blocking solution.

Approximately 4 hours later:
Place slide in blocking solution
Wash with BSA/Triton/TBS solution.
Add antibody to slide sandwiches (about 30min for 10 slide pairs)
Incubate at 4°C overnight.

Day 3
Wash excess antibody off the slides

approximately 2 hours later:
Set up the colour reaction
Add the alkaline phosphate substrate to the sandwiched slides (about 30min for 10 slide pairs)
Leave in a sealed box above damp towels in the dark at room temperature for 1-5 days.