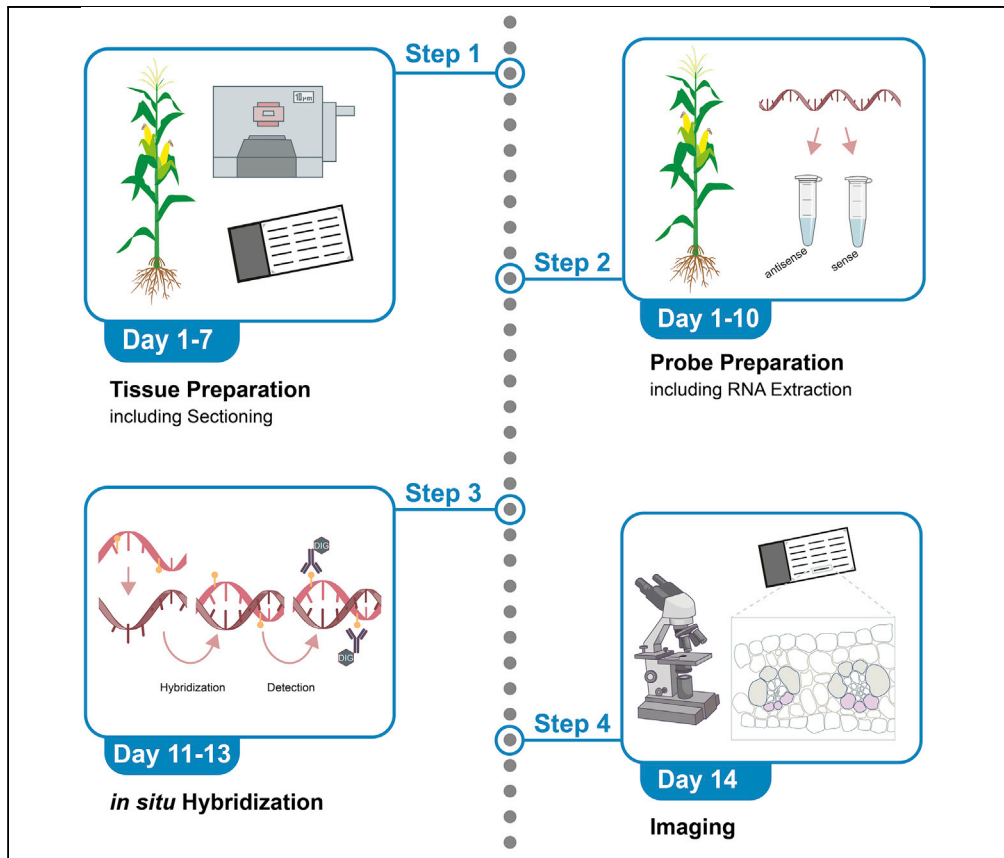


Protocol

An RNA *in situ* hybridization protocol optimized for monocot tissue



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Highlights
Optimized for
monocot tissues and
tested on maize,
sorghum, and
teosinte

Clear results with low
background and high
resolution

Three unique samples
for each gene of
interest increase
specificity

Comprehensive
protocol with
detailed explanation
of all necessary steps

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Protocol

An RNA *in situ* hybridization protocol optimized for monocot tissue

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SUMMARY

RNA *in situ* hybridization can be time-consuming and difficult to troubleshoot. Here, we provide an optimized protocol for maize leaf tissue, though it can be applied to other plant tissues such as shoot apical meristems, embryos, and floral organs. We generate three >100 bp unique antisense probes for each gene of interest and hybridize them to tissue sections.

For complete details on the use and execution of this protocol, please refer to Bezruczyk et al. (2021).

BEFORE YOU BEGIN

This protocol is optimized for monocot tissue based on protocols from Jackson and Kellogg labs (Jackson, 1991; Jackson et al., 1994; Malcomber and Kellogg, 2004). The results are clearer, have less background and the protocol is therefore easier to troubleshoot, which was very complicated before (Stahl and Simon, 2010). It was primarily applied to the most prominent model organism among the monocots, *Zea mays*. Besides, the application to other organisms, such as *Sorghum bicolor* and *Euchlaena mexicana*, have successfully been performed (unpublished). The authors see no reason to doubt the suitability of this protocol for other monocots.

Solutions that can be prepared in advance

Timing: ~1 day

Specific recipes for each reagent (Table 1) are found under ‘material and equipment’.

Solutions that have to be freshly prepared

Specific recipes for each reagent (Table 2) are found under “materials and equipment”.



KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-digoxigenin AP-conjugated antibody	Merck/Roche	Cat#11333062910
Bacterial and virus strains		
One Shot TOP10 Chemically Competent <i>E. coli</i>	Thermo Fisher	Cat#1096176
Chemicals, peptides, and recombinant proteins		
50× Denhardt's solution	Sigma-Aldrich	Cat#D2532
Boehringer block reagent	Merck/Roche	Cat#1096176
DEPC	Carl Roth	Cat#K028.3
Proteinase K	Thermo Fisher	Cat#EO0491
TRNA	Merck/Roche	Cat#10109517001
Critical commercial assays		
CloneJET PCR cloning kit	Thermo Fisher	Cat#K1232
DIG labeling kit	Merck/Roche	Cat#11277073910
NucleoSpin Gel and PCR Clean-up kit	Macherey-Nagel	Cat#740609.50
PrimeSTAR GXL polymerase	Takara Bio	Cat#R050A
QuantiTect Reverse Transcription Kit	Qiagen	Cat#205311
The MEGAscript SP6 Transcription kit	Thermo Fisher	Cat#Am1330
Software and algorithms		
Geneious R11	N/A	www.geneious.com
Other		
BCIP	Merck/Roche	Cat#11383221001
Histo-Clear	Thermo Fisher	Cat#12358637
Hot plate	VWR	Cat#442-0185
Hybridization chamber	Merck	Cat#Z670146-1EA
HybriSlip hybridization coverslip	Thermo Fisher	Cat#10625983
Microtome	Leica	RM 2255 replaced Cat#14051956472
NBT	Merck/Roche	Cat#11383213001
Paraplast	Carl Roth	Cat#X880.1
ProbeOn Plus slides	Thermo Scientific (not available in Europe)	Cat#15-188-51
RNase-ExitusPlus	PanReac AppliChem	Cat# A7153
ROCKER 3D digital	IKA	Cat#0004001000
Sectioning cartridge	Thermo Scientific	Cat#12740908100957
Slide box for long-time storage	VWR	Cat#631-0735
Slide dryer	Plano	Cat#12857-220-D
Slide rack with container	Merck	Cat#BR471800-5EA
SuperFrost Ultra Plus GOLD Adhesion slides	Thermo Scientific	Cat#11976299
Water bath	Plano	Cat#28147-220

Alternatives: ProbeOn Plus slides are not available in Europe. The integrated spacer function allows the user to make slide sandwiches consisting of two slides facing each other rather than a single slide with a coverslip. This saves time and reagents because the same volume of probe and hybridization solution is used to treat twice as many slides. As an alternative, SuperFrost Ultra Plus GOLD Adhesion slides can be used in combination with HybriSlip Hybridization coverslips.

Alternatives: PrimeSTAR GXL polymerase gives consistently good results but can be replaced by your preferred polymerase.

Alternatives: Geneious R11 can be replaced by any other software supporting cloning, primer design and sequencing analysis.

MATERIALS AND EQUIPMENT

50% Dextran sulfate

Reagent	Final concentration	Amount
Dextran sulfate	50% w/v	25 g
RNase-free water	n/a	to 50 mL
Total		50 mL

Note: Dissolve by gently rocking over night at 55°C. Centrifuge to remove bubbles and mix for another 2 to 3 h. Store at –20°C for long-time storage.

tRNA

Reagent	Final Concentration	Amount
tRNA	100 mg/mL	100 mg
RNase-free water	n/a	1 mL
Total		1 mL

Note: Make 2 aliquots of 500 µL. Store at –20°C for long-time storage.

0.5 M EDTA pH 8.0

Reagent	Final concentration	Amount
EDTA	500 mM	14.6 g
DEPC water	n/a	to 100 mL
Total		100 mL

△ **CRITICAL:** For any solutions that contain EDTA, the DEPC water should be prepared first in bulk and then used to prepare the solutions, because EDTA chelates DEPC.

Note: Stir the solution for 2 h at 37°C. Adjust pH with HCl. Store at 22°C ± 2°C for long-time storage.

10× *in situ* salts

Reagent	Final concentration	Amount
NaCl	3000 mM	8.78 g
NaH ₂ PO ₄ ·H ₂ O	50 mM	0.39 g
Na ₂ HPO ₄	50 mM	0.355 g
Tris-HCl pH 6.8 (1 M)	100 mM	5 mL
EDTA pH 8 (0.5 M)	50 mM	5 mL
RNase-free water	n/a	to 50 mL
Total		50 mL

Note: Store at –20°C for long-time storage .

10% Acetic Acid

Reagent	Final concentration	Amount
Glacial acetic acid	10% v/v	2 mL
RNAse-free water	n/a	to 20 mL
Total		20 mL

Note: Store at 22°C ± 2°C for long-time storage.

Hybridization solution

Reagent	Final concentration	Amount
10× <i>in situ</i> salts	n/a	5 mL
Formamide	20 mM	20 mL
50% Dextran sulfate	n/a	10 mL
tRNA 100 mg/mL	n/a	0.5 mL
50× Denhardt's solution	n/a	1 mL
RNAse-free water	n/a	to 40 mL
Total		40 mL

Note: Keep solution RNAse-free and store at –20°C for long-time storage. Details to 50× Denhardt's solution can be found in [key resource table](#).

△ **CRITICAL:** Handle formamide in a fume hood.

10× PBS pH 7.4

Reagent	Final concentration	Amount
NaCl	1750 mM	102.2 g
Na ₂ HPO ₄	84.1 mM	11.94 g
NaH ₂ PO ₄ ·H ₂ O	18.6 mM	2.56 g
ddH ₂ O	n/a	to 1 L
Total		1 L

Note: Adjust pH with HCl. Store at 22°C ± 2°C for long-time storage.

20× SSC pH 7.0

Reagent	Final concentration	Amount
NaCl	3000 mM	175.3 g
Sodium citrate	340 mM	88.2 g
ddH ₂ O	n/a	to 1 L
Total		1 L

Note: Adjust pH with HCl. Store at 22°C ± 2°C for long-time storage.

1 M Tris-HCl pH 6.8

Reagent	Final concentration	Amount
Tris	1000 mM	121 g
ddH ₂ O	n/a	to 1 L
DEPC	n/a	1 mL
Total		1 L

Note: Stir the solution for 2 h at 37°C and adjust pH with HCl. Autoclave afterward to inactivate DEPC. Store at 22°C ± 2°C for long-time storage.

1 M Tris-HCl pH 7.5

Reagent	Final concentration	Amount
Tris	1000 mM	121 g
ddH ₂ O	n/a	to 1 L
DEPC	n/a	1 mL
Total		1 L

Note: Stir the solution for 2 h at 37°C and adjust pH with HCl. Autoclave afterward to inactivate DEPC. Store at 22°C ± 2°C for long-time storage.

1 M Tris-HCl pH 9.5

Reagent	Final concentration	Amount
Tris	1000 mM	121 g
ddH ₂ O	n/a	to 1 L
DEPC	n/a	1 mL
Total		1 L

Note: Stir the solution for 2 h at 37°C and adjust pH with HCl. Autoclave afterward to inactivate DEPC. Store at 22°C ± 2°C for long-time storage.

5 M NaCl

Reagent	Final concentration	Amount
NaCl	5 M	292 g
ddH ₂ O	n/a	to 1 L
Total		1 L

Note: Store at 22°C ± 2°C for long-time storage.

1 M MgCl₂

Reagent	Final concentration	Amount
MgCl ₂	1 M	95 g
ddH ₂ O	n/a	to 1 L
Total		1 L

△ **CRITICAL:** Paraformaldehyde is harmful to health. Work in a fume hood when handling paraformaldehyde solutions and wear a protective mask when weighing PFA powder.

Note: Store at 22°C ± 2°C for long-time storage.

4% Paraformaldehyde-Triton pH 7.0

Reagent	Final concentration	Amount
PFA	200 mM	16 g
30% Triton-X100	0.03%	400 µL
1× PBS pH 7.4	n/a	to 400 mL
Total		400 mL

Note: Add 3 NaOH pellets to dissolve PFA in 350 mL, then adjust pH to 7.0 with HCL; top up with PBS to 400 mL. Needs to be prepared freshly.

Note: Prepare 4% Paraformaldehyde (without triton) likewise, but without Triton-X100.

Proteinase K buffer

Reagent	Final concentration	Amount
Tris-HCl pH 7.5 (1 M)	100 mM	30 mL
EDTA pH 8 (0.5 M)	10 mM	6 mL
ddH ₂ O	n/a	to 300 mL
Total		300 mL

Note: Needs to be prepared freshly.

0.2% Glycine

Reagent	Final concentration	Amount
Glycine	0.2% w/v	0.6 g
1× PBS pH 7.4	n/a	to 300 mL
Total		300 mL

Note: The solution can be reused four to five times. Store at 22°C ± 2°C for this period.

0.1 M Triethanolamine pH 8.0

Reagent	Final concentration	Amount
Triethanolamine	100 mM	4.125 mL
ddH ₂ O	n/a	to 300 mL
Total		300 mL

Note: Triethanolamine is very viscous: pipette slowly and carefully. Needs to be prepared freshly. Adjust pH to 8.0 with HCl.

Buffer C

Reagent	Final concentration	Amount
Tris-HCl pH 9.5 (1 M)	100 mM	10 mL
NaCl (5 M)	100 mM	2 mL
MgCl ₂ (1 M)	50 mM	5 mL
ddH ₂ O	n/a	to 100 mL
Total		100 mL

Note: Needs to be prepared freshly.

Block solution 1

Reagent	Final concentration	Amount
Boehringer Blocking reagent	n/a	0.5 g
Tris-HCl pH 7.5 (1 M)	100 mM	5 mL
NaCl (5 M)	150 mM	1.5 mL
ddH ₂ O	n/a	to 50 mL
Total		50 mL

Note: Dissolve at 60°C–70°C for at least 1 h. Details to Boehringer Blocking reagent can be found in [key resource table](#). Needs to be prepared freshly.

BSA block solution 2

Reagent	Final concentration	Amount
Bovine Serum Albumin	n/a	3 g
Tris-HCl pH 7.5 (1 M)	100 mM	30 mL
NaCl (5 M)	150 mM	9 mL
30% Triton-X-100	0.09%	0.9 mL
ddH ₂ O	n/a	to 300 mL
Total		300 mL

Note: Needs to be prepared freshly.

STEP-BY-STEP METHOD DETAILS

Tissue preparation

⌚ Timing: 5 days

In this section plant samples are harvested and prepared for sectioning. The goal of this step is to create fixed, cleared samples that are embedded in Paraplast.

Harvesting and fixation

Note: Perform the steps under a fume hood.

1. Add roughly 10 mL of PFA-Triton-X into a glass scintillation vial and keep on ice.

Table 1. Solutions that can be prepared in advance and information about their treatment to achieve sterile and/or RNase-free conditions

REAGENT	Volume	Sterilization method	RNase-free
1 M Tris-HCl pH 6.8	1 L	Autoclave	DEPC water
1 M Tris-HCl pH 7.5	1 L	Autoclave	DEPC water
1 M Tris-HCl pH 9.5	1 L	Autoclave	DEPC water
0.5 M EDTA pH 8	100 mL	Autoclave	DEPC water
5 M NaCl	500 mL	Autoclave	DEPC water
1 M MgCl ₂	500 mL	n/a	n/a
50% Dextran Sulfate	50 mL	n/a	DEPC water
100 mg/mL tRNA	1 mL	n/a	DEPC water
10% Acetic Acid	20 mL	n/a	DEPC water
10× <i>in situ</i> salts	50 mL	Filter sterilize	DEPC water
20× SSC pH 7	1 L	n/a	DEPC water
10× PBS pH 7.4	1 L	Autoclave	n/a
1× PBS pH 7.4	1 L	n/a	n/a
50× TAE	1 L	Autoclave	n/a
1× TAE	1 L	n/a	n/a
1% (w/v) agarose in 1×TAE buffer	500 mL	n/a	n/a
50% Formamide	50 mL	n/a	DEPC water
Hybridization solution	40 mL	n/a	DEPC water
15, 30, 50, 70, 80, 90% ethanol	500 mL each	n/a	n/a
25, 50, 75, 100% Histo-Clear in ethanol	500 mL each	n/a	n/a
LB-Carbenicillin plates	n/a	n/a	n/a

Specific recipes for each reagent are found under “[materials and equipment](#)”.

Alternatives: Any other small container which allows the tissue to lie flat can be used.

2. Cut a piece of the leaf tissue from a healthy plant and directly after cutting, place it into the fixative.
 - a. The size of the segment is relevant for penetration of the fixative and clearing agents.
 - b. One dimension of the tissue has to be less than 1 mm which is the leaf thickness in this case. We recommend a size of (LxWxH) 1 cm x 1 cm x 1 mm.
 - c. Each vial can carry up to 4 pieces of leaf tissue.
3. Place the scintillation vial on ice under a vacuum bell and pull a vacuum for 10 min.
4. Carefully release the vacuum. Repeat the step two to five times until sinking of the tissue is observed.
5. Replace with fresh fixative. Leave 12 to 14 h at 4°C.

Pause point: The tissue may be stored in 70% or 100% ethanol for longer periods of time at 4°C.

Dehydration

Note: Perform the steps on a rocker at 30 rpm.

6. Incubate in 1× PBS for 1 h on ice.
7. Incubate in 1× PBS for 1 h on ice.
8. Incubate in 15% ethanol for 1 h on ice.
9. Incubate in 30% ethanol for 1 h on ice.
10. Incubate in 50% ethanol for 1 h on ice.
11. Incubate in 70% ethanol for 1 h on ice.
12. Incubate in 80% ethanol for 1.5 h on ice.

Table 2. Solutions that have to be prepared fresh and information about their treatment to achieve sterile and/or RNase-free conditions

REAGENT	Volume	Sterilization method	RNase-free
4% PFA-Triton in PBS pH 7.0	400 mL	n/a	n/a
4% PFA in PBS pH 7.0	400 mL	n/a	n/a
Proteinase K buffer pH 7.5 at 37°C	300 mL	n/a	n/a
0.2% Glycine solution	300 mL	n/a	n/a
0.1 M Triethanolamine	300 mL	n/a	n/a
0.2× SSC 12 to 14 h at 55°C (for day 2)	1 L	n/a	n/a
Block solution 1	50 mL	n/a	n/a
BSA block solution 2	300 mL	n/a	n/a
Buffer C	100 mL	n/a	n/a

13. Incubate in 90% ethanol for 1.5 h on ice.
14. Incubate in 100% ethanol for 1.5 h on ice.
15. Incubate in 100% ethanol for 12 to 14 h at 4°C.

Note: The tissue should slowly de-stain and chlorophyll should be visible in the ethanol.

▮▮ Pause point: Dehydrated tissue may be stored in 100% ethanol at 4°C for 1 to 3 days.

Clearing

Note: Perform the steps on a rocker under a fume hood. Details to Histo-Clear can be found in [key resource table](#).

16. Incubate in 100% ethanol for 2 h at 22°C ± 2°C.
17. Incubate in 25% Histo-Clear/75% EtOH for 1 h at 22°C ± 2°C .
18. Incubate in 50% Histo-Clear/50% EtOH for 1 h at 22°C ± 2°C .
19. Incubate in 75% Histo-Clear/25% EtOH for 1 h at 22°C ± 2°C .
20. Incubate in 100% Histo-Clear for 1 h at 22°C ± 2°C .
21. Incubate in 100% Histo-Clear for 1 h at 22°C ± 2°C .
22. Incubate in 100% Histo-Clear for 1 h at 22°C ± 2°C .

Embedding and plate pouring

△ CRITICAL: Paraplast consists of paraffin wax (see [key resource table](#)), plastic polymers and DMSO. Paraplast solidifies between 56°C–57°C but can degrade above 62°C (Costa, 2018). Be careful to not heat Paraplast higher than 60°C and avoid re-melting, if possible (Jackson, 1991).

23. Add Paraplast chips by half of the vial and fill it up with Histo-Clear. Leave it 12 to 14 h at 60°C.
24. Also, place a 500 mL bottle fully filled with Paraplast chips to 60°C and leave it 12 to 14 h to melt.
25. For three days in a row replace the vial content with freshly molten Paraplast in the morning and evening and leave at 60°C in between.
26. Quickly pour the complete contents of the scintillation vial into a plastic petri dish.
27. The wax will solidify before the samples can be arranged. Move the dish to a hot plate (<60°C) and let the Paraplast melt completely.
28. Using preheated forceps, gently remove any air bubbles from above and underneath the plant tissue.
29. Arrange the sections in the petri dish with maximum distance between them (Figure 1A).
30. Remove the dish from the hot plate and let the Paraplast solidify at 22°C ± 2°C for ~1 h.

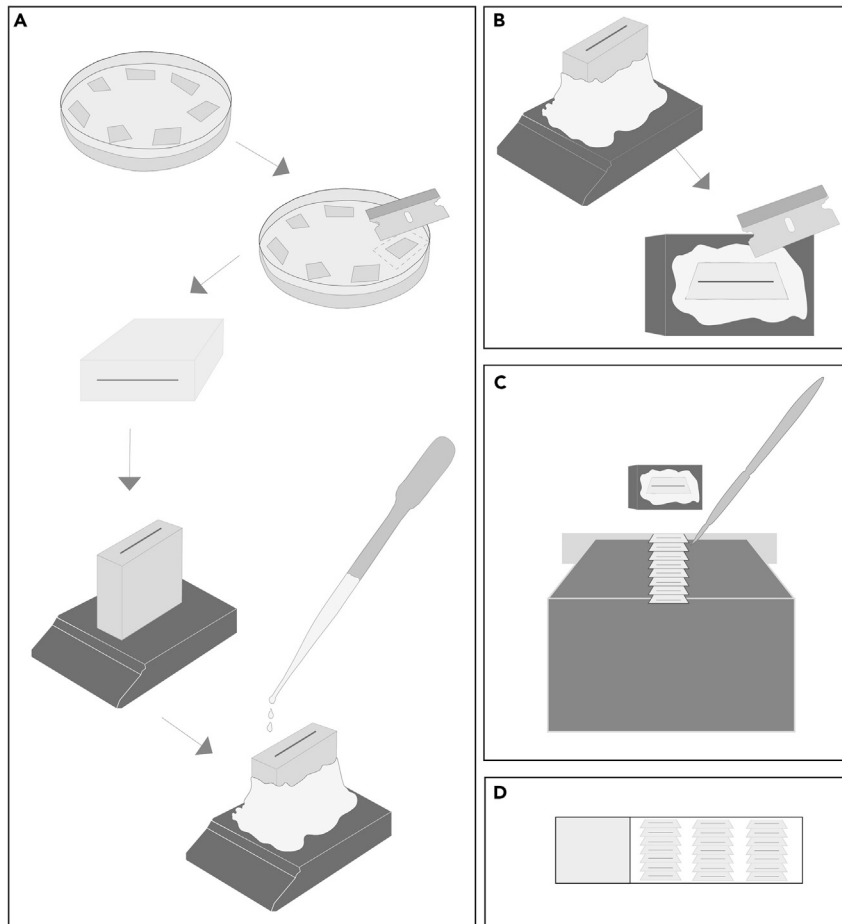


Figure 1. Sample mounting and sectioning

(A) Embedded tissue in liquid Paraplast is poured into a petri dish, where a wax block is cut out after arranging and solidifying and mounted on a sample cartridge with fresh Paraplast as support structure.

(B) The mounted wax block is trimmed into a trapezoidal prism shape using a razor blade.

(C) Ribbons are produced by microtome sectioning the wax block, which is arranged with the long side touching the blade first. A good-looking ribbon is transferred to the surface of a water bath by detaching it from the blade also with a paint brush.

(D) Three ribbons with the length of the slide height are aligned close to each other to fill as much of the slide as possible.

Note: Solidified Paraplast easily breaks in an uncontrolled manner. Mark predetermined breaking points at the surface by scoring it with a razor blade (Figure 1A).

Sectioning

⌚ Timing: 2 days

Sample mounting

Note: We prefer to mount the sample in two steps to make selecting the correct orientation of the final sections easier. The orientation is highly dependent on the experiment and should be considered carefully.

31. Warp the petri dish to carefully loosen the solidified plate of wax out of the plastic. The sides can be separated from the dish by a razor blade.
32. Cut out one single plant tissue as a wax block in a rectangular shape (Figure 1A).
33. It is important not to cut directly at the tissue but to keep a surrounding wax layer of 2–5 mm.
34. In a separate dish, melt Paraplast on the hot plate (<60°C).

Note: Molten Paraplast can be handled with disposable transfer pipets.

35. Place the wax block in the desired orientation on a sectioning cartridge and add molten Paraplast to the sample holder and onto the sample sides building surrounding support structure. The mount will be stronger if Paraplast is added to the back of the cartridge as well (Figure 1A). The sample can also be poured into a mold, but we find it is easier to obtain the correct orientation with this method.
36. Let the mounted sample harden at 22°C ± 2°C for ~1 h.

Pause point: Embedded tissue in petri dishes or on sample holders may be stored at 22°C ± 2°C for several years.

Microtome sectioning

Note: Paraffin sectioning takes time and patience to learn. To prepare high quality sections, a calm state of mental clarity is essential.

37. Heat the slide dryer and water bath to 37°C–42°C.
38. Place the mounted sample on ice until use.
39. Use a razor blade to trim the sample before securing it to the microtome sample holder. A trapezoidal prism shape is best, with the long side striking the blade first; this will facilitate the formation of a continuous ribbon of sections for easy handling (Figure 1B).
40. Use the handwheel to bring the sample to the same height as the blade. Carefully position the blade as close as possible to the sample and fix the blade position.
41. Orient the sample parallel to the blade with the setscrews (Figure 1C).
42. To remove the first layer of paraffin without any sample and to generate an even sample surface, trim it by choosing a cutting thickness of ~60 μm.
 - a. Thick sections such as these will form rolls of Paraplast.
 - b. Trimming can be stopped when the paraffin rolls have a uniform width at both sides.
43. Change the operating mode from trimming to sectioning.
 - a. Set a sectioning of 10 μm.
44. Cut Paraplast ribbons of your sample (Figure 1C). [Troubleshooting 1](#)
 - a. The ribbon should have the length of the slide width.
 - b. The number of sections per ribbon therefore depends on the single section thickness and changes with every sample (Figure 1D).
45. Loosen the ribbon from the blade by carefully detaching it with a paint brush (Figure 1C).
46. Use the paint brush to transfer the ribbon to the water surface. [Troubleshooting 1](#).
 - a. This will flatten the section.
 - b. If the paraffin sticks to the brush, a graphite pencil tip can help to detach.

Note: Use ProbeOn Plus slides if possible (see [key resource table](#)).

47. Hold the slide at the labeling end with one hand and dip it at an angle into the water bath. The slide should be nearly completely under water.
48. Use the other hand to bring one section by the other to the slide with a paint brush. Arrange it with the brush on the water surface over the slide in the desired orientation and carefully lift the slide to fix there.
49. Continue with all remaining ribbons.

- a. Try to move as many ribbons onto the slide as possible for maximal space usage (Figure 1D).
50. Drain off excess water with a Kim wipe from underneath the slide and place them on the slide dryer for 12 to 14 h.

▮▮ Pause point: If slides are not immediately needed, they may be stored in a slide box for several weeks at 4°C.

Probe preparation

⌚ **Timing:** 7–10 days

Depending on the probe design, probes are generated from either gDNA or cDNA. In general probes can be cloned from gDNA, which can be prepared using standard DNA extraction protocols. If probes target cDNA regions spanning introns or if specific probes targeting isoforms of a gene are necessary, it is recommended to clone probes from cDNA. In this section leaf RNA is extracted and reverse transcribed into cDNA. RNA sense and antisense probes are designed and produced. Here, this protocol differs from other protocols in that we recommend you generate several unique and specific probes for each gene.

Probe design

Note: For each gene two to three probes will be designed to cover different regions of the gene. The specification of those as antisense and sense probe will occur later during transcription.

51. We recommend designing three independent probes of approximately 100 bp each in the 5'-UTR, 3'-UTR, and in at least one other location (Figures 2B and 2C).
52. As a general point, it is important to consider whether there are multiple isoforms of your gene of interest, which should be aligned first to ensure that the probe binds to all transcripts, unless the purpose of the experiment is to target a specific isoform of a gene.
53. If there are many genes with high sequence homology to your gene of interest, the probe template regions must be carefully selected to avoid designing a non-specific probe, and this may limit the number of probes per gene if three unique 100 bp regions are not available.
54. We recommend using one sense probe for each antisense probe as a negative control, as these have identical GC content and length to the antisense probes, and may be required by journal editors or reviewers for publication. Alternatively, one could generate random sequence controls.

RNA extraction for probe production

Alternatives: The procedures for performing RNA extraction are included for completeness. We recommend this protocol, however, any established RNA extraction protocol can be used.

⚠ CRITICAL: Maintain RNase-free conditions.

⚠ CRITICAL: Phenol and chloroform are toxic. Work under a fume hood and wear a lab coat, safety goggles and appropriate gloves.

55. Prepare RNA buffer with 100 mM sodium acetate, 1 mM sodium-EDTA and 4% (w/v) SDS.
56. Prepare acid (pH 4.7) phenol:chloroform:isoamylalcohol 25:24:1. This mixture must sit until fully cleared, which can take up to an hour.
57. Mix RNA buffer with phenol:chloroform:isoamylalcohol in 1:1 (v/v) ratio to prepare the RNA extraction solution.
58. Place a bottle of 8M LiCl and bottle of 80% ethanol at –20°C.

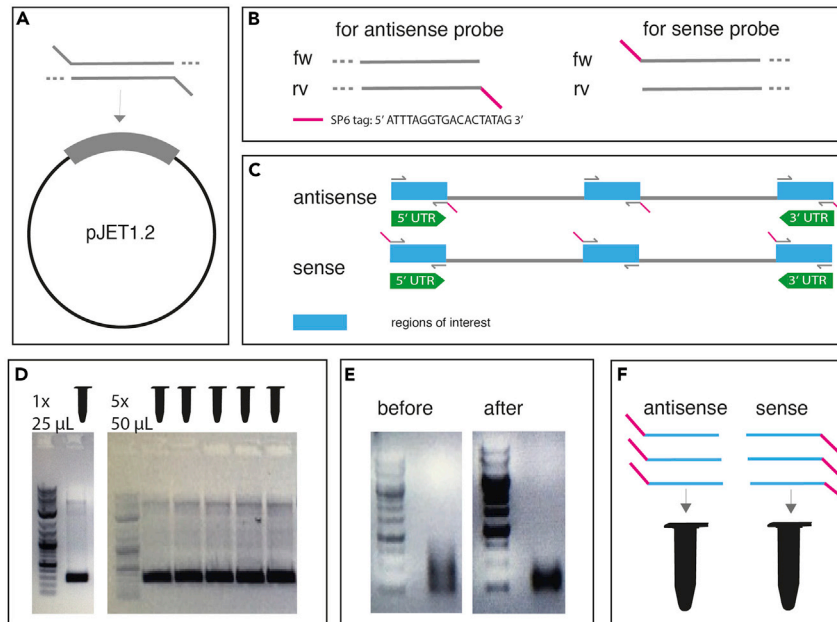


Figure 2. Probe design, cloning, and generation

(A) Ligation of the gene of interest into the pJET1.2 vector using the CloneJet PCR cloning kit.
 (B) Schematic design of SP6-tagged probe template primers.
 (C) Schematic design of hybridization probes from three different regions of interest. One region is located in the 5'-UTR, one in the middle and one in the 3'-UTR.
 (D) Assembly of small and big probe template PCRs and their product purification from an agarose gel after agarose gel electrophoresis. GeneRuler 1 kb serves as DNA ladder.
 (E) After SP6 transcription over night the products of sense and antisense probes can be differentiated by their location of the tag. The removal of the remaining DNA from the DNA template is supervised by two agarose gel electrophoreses containing DNase un- and treated transcription product. GeneRuler 1 kb serves as DNA ladder.
 (F) The 5'-UTR, coding region, and 3'-UTR RNA probes can be mixed in equal amounts to generate sense and antisense probe mixes.

⚠ **CRITICAL:** Plant tissue must remain frozen. Pre-cool spatulas, mortar and pestles, and tubes with liquid nitrogen.

⚠ **CRITICAL:** Working with liquid nitrogen has to be performed carefully while wearing a lab coat, safety goggles and appropriate gloves.

59. Homogenize plant tissue using mortar and pestle in liquid nitrogen. Transfer about 300 mg of powder to a pre-cooled 2 mL tube.
60. Aliquot 1.5 mL RNA extraction solution, add ground tissue powder, and vortex immediately.
61. Incubate at 58°C (< 60°C) and vortex every few minutes during the 30 min incubation time.

⚠ **CRITICAL:** During the incubation chloroform evaporation may cause the tube to explode if heated above 58°C: handle with care and wear PPE.

62. Centrifuge samples for 10 min at 14.000 × g and 22°C ± 2°C.
63. Transfer the top phase to a new tube and add 950 µL phenol:chloroform:isoamylalcohol (pH 4.7). Vortex under a fume hood.
64. Centrifuge samples for 5 min at 14.000 × g and 22°C ± 2°C.
65. Transfer the top phase to a new tube and add 950 µL phenol:chloroform:isoamylalcohol (pH 4.7). Vortex under a fume hood.

66. Centrifuge samples for 5 min at $14.000 \times g$ and $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$.
67. Carefully transfer the top phase into a new 2 mL tube.
68. Centrifuge samples for 5 min at $14.000 \times g$ and $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$.
69. Carefully transfer the top phase into a new 2 mL tube and add 0.5 volumes of ice-cold 8M LiCl.
70. Leave RNA at -20°C to precipitate for at least 2 h; 12 to 14 h results in better yield.
71. Centrifuge samples for 15 min at $14.000 \times g$ and 4°C .
72. Remove the supernatant.
73. Add 1 mL of ice-cold 80% ethanol to the tube and invert; pellet becomes opaque.
74. Centrifuge samples for 2 min at $14.000 \times g$ and $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$.
75. Remove the supernatant.
76. Add 1 mL of ice-cold 80% ethanol to the tube and invert.
77. Centrifuge samples for 2 min at $14.000 \times g$ and $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$.
78. Carefully remove as much ethanol as possible and air dry the pellet under a sterile bench until the pellet edges become clear, but no longer than 5 min.
79. Dissolve the pellet in 20–40 μL RNA elution buffer.
80. Either store RNA at -80°C , leave at 4°C for analysis or keep on ice for cDNA synthesis.

cDNA synthesis and cloning

81. Synthesize cDNA from your isolated RNA using the QuantiTect Reverse Transcription kit and its enclosed random hexamers primer mix (see [key resource table](#)).
82. Amplify the gene of interest from cDNA with Takara PrimeSTAR GXL DNA Polymerase.
83. Ligate the PCR product into pJET1.2 following instructions of CloneJet PCR cloning kit ([Figure 2A](#)).
84. Transform chemically competent TOP10 cells with the ligation product and plate on carbenicillin selection plates.
85. Perform a colony PCR with a standard Taq polymerase on at least 5 colonies.
86. Isolate plasmids containing the gene of interest with the NucleoSpin Plasmid kit.
87. Sequence the pJET plasmids containing your gene of interest and continue with the protocol if results match expectations.

Template PCR

88. Amplify the regions of interest you selected using PrimeSTAR GXL DNA polymerase to generate a DNA template for RNA probe synthesis.
89. Perform one small test probe template PCR (preliminary PCR) ($1 \times 25 \mu\text{L}$) in advance to verify that PCR settings are successful, and the reaction is specific ([Figure 2D](#)).
90. Perform two large (we recommend $5 \times 50 \mu\text{L}$) probe template PCR reactions for each region of interest, one for the antisense and the other for the sense probe:
 - a. For the antisense probe: Perform a PCR with corresponding SP6-tagged primers on the reverse primer ([Figure 2B](#)).
 - b. For the sense probe: Perform a PCR with corresponding SP6-tagged primers on the forward primer ([Figure 2B](#)).
91. If several DNA fragments visible in the preliminary PCR:
 - a. Load total PCR product ($5 \times 50 \mu\text{L}$) onto a 1% agarose gel prepared with $1 \times$ TAE buffer. Separate DNA fragments at 100 V and 400 mA for 40 min ([Figure 2D](#)).
 - b. Excise the fragment of interest and purify with a dedicated NucleoSpin Gel and PCR Clean-up kit for RNase-sensitive experiments.
 - c. Use preheated (70°C) RNase-free water to elute and maintain RNase free environment from this step forward.
92. If one DNA fragment visible in the preliminary PCR:
 - a. Purify total PCR product ($5 \times 50 \mu\text{L}$) with NucleoSpin Gel and PCR Clean-up kit.

- b. Use preheated RNase-free water to elute and maintain RNase free environment from this step forward.

SP6 transcription

93. Estimate the dsDNA concentration of the purified probe template using a spectrophotometer.
94. Calculate the final volume needed to reach a concentration of 500 ng/ μ L.
95. Place the open tube under a sterile bench and evaporate until the desired volume and concentration is reached. This can take up to several hours, depending on the initial concentration.
96. Perform a transcription reaction using the concentrated probe template as described in the manual of the MEGAscript SP6 transcription kit, using DIG RNA Labeling Mix (1:3) with kit-supplied rNTPs (see [key resource table](#)). This reaction is performed 12 to 14 h at 37°C ([Figure 2E](#)).

Note: Do not use a heat block for this reaction, as the small reaction volume may result in complete evaporation and condensation on the lid within a 1 mL tube. Place the entire tube in a 37°C oven or chamber.

Probe purification

97. Save 0.2 μ L in a separate tube to visualize on an agarose gel, then add 1 μ L TURBO DNase (included in the MEGAscript SP6 transcription kit) to the remaining transcription RNA probe.
98. Incubate for 20 min at 37°C.
99. To stop the DNA digestion, add 24.6 μ L RNase-free water and 0.4 μ L 0.5 M EDTA. Incubate for 10 min at 75°C.
100. Again, save 0.2 μ L of TURBO DNasetreated RNA probe for agarose gel imaging.
101. Perform a quick agarose gel electrophoresis with both samples: 1% agarose gel, 5 min, 100 V and 400 mA ([Figure 2E](#)).
102. Fragments should appear as discrete bands of the expected size in both gels: if so, continue with purification. If not, repeat SP6 transcription. To purify, treat your sample with the following solutions:
 - a. 2 μ L 2 mg/ml glycogen
 - b. 4 μ L 10% acetic acid
 - c. 4 μ L 3 M NaOAc
 - d. 96 μ L 100% ethanol

Note: It is possible that the RNA forms secondary and tertiary structures, preventing efficient migration through the gel. This is typically visualized by multiple bands or a smear above the expected discrete band.

103. Centrifuge the reaction tube for 30 min at 20,000 \times g and 4°C.
104. Carefully remove the supernatant and wash the pellet with 70% ethanol in RNase-free water at 22°C \pm 2°C.
105. Decant and dry pellet for 5 min under sterile hood.

△ CRITICAL: Handle formamide in a fume hood.

106. Resuspend the pellet in 25 μ L RNase-free 10 mM TE buffer pH 8 and 25 μ L formamide.
107. Store RNase safe at -20°C.

In situ hybridization

⌚ Timing: min 2 days

In this step, your sectioned tissue is first pretreated to make it accessible for the RNA probes. Then the hybridization is performed and the detection process will be initiated. At the end of this step your sample will be ready to image.

Note: It is paramount to maintain an RNase-free environment. We recommend cleaning the bench, pipettes, tip boxes etc. with Rnase-ExitusPlus or other similar treatment. Wear gloves and change them frequently. Wear a dedicated lab coat that is only for RNA *in situ* hybridization. The solutions used in this step should be exclusively for RNA *in situ* hybridization. Most can be reused several times, except for the 100% ethanol, PFA, proteinase K buffer, PBS and HistoClear.

Note: To treat the sections on the slides we recommend placing 18 or any other even number of the slides into the slide rack (see [key resource table](#)). Use empty, clean 1000 μ L tip boxes, which fit the slide rack. Move the rack from box to box. Use a volume of solution that covers the slides completely: we recommend 300 mL. Incubations can be performed on an orbital shaker. The given incubation times are minimums, except for the proteinase K, which should not incubate longer than 10 min. Perform all steps at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ unless otherwise stated.

Deparaffinization and rehydration

108. Incubate in Histo-Clear for 10 min.
109. Incubate in Histo-Clear for 10 min.
110. Incubate in 100% ethanol for 30 s.
111. Incubate in 100% ethanol for 30 s.
112. Incubate in 90% ethanol for 30 s.
113. Incubate in 80% ethanol for 30 s.
114. Incubate in 70% ethanol for 30 s.
115. Incubate in 50% ethanol for 30 s.
116. Incubate in 30% ethanol for 30 s.
117. Incubate in H₂O for 30 s.
118. Incubate in 1 \times PBS for 2 min.

Permeabilization

119. Add 30 μ L proteinase K (20 mg/mL) to 300 mL pre-warmed proteinase K buffer.
120. Incubate slides for 10 min in the proteinase K-containing buffer at 37C.
121. Incubate in 0.2% glycine solution for 2 min.
122. Incubate in 1 \times PBS for 2 min.

Refixation

123. Incubate in 4% PFA for 10 min.
124. Incubate in 1 \times PBS for 2 min.

Acetylation

△ CRITICAL: This is a unique but critical step for this *in situ* hybridization protocol. Acetylation reduces non-specific binding of the probe to the tissue and reduces background.

125. Incubate in 0.1 M triethanolamine while dribbling 1.5 mL acetic anhydride over the slides.
126. If possible, suspend the slide rack above a stir bar and place its container on a stirring plate, so that the solutions can be constantly mixed.
127. Incubate for 10 min.

128. Incubate in 1× PBS for 2 min.

Dehydration

129. Incubate in 30% ethanol for 30 s.
130. Incubate in 50% ethanol for 30 s.
131. Incubate in 70% ethanol for 30 s.
132. Incubate in 80% ethanol for 30 s.
133. Incubate in 90% ethanol for 30 s.
134. Incubate in 100% ethanol for 1 min.
135. Incubate in 100% ethanol for 2 min.

Pause point: At this point it is possible to store the slides at 4°C for up to several days. Therefore, keep the slide rack in the tip box filled with a small amount of ethanol to cover the bottom and seal the box with Parafilm.

Hybridization

Note: Decide on the probe concentration for the hybridization solution. For new probe, use multiple concentrations ranging from 0.5× to 2×.

136. Thaw the hybridization solution at 22°C ± 2°C, set an oven to 55°C, and a heat block to 98°C.

△ **CRITICAL:** Handle formamide in a fume hood.

137. Thaw all RNA sense and antisense probes on ice and fill hybridization chamber (see [key resource table](#)) with 100 mL formamide and 100 mL H₂O.
138. Remove slides from ethanol by arranging them on a fresh paper towel. Place slide pairs next to each other, whereby a slide pair is considered to be two slides that will be treated with the same probe. If you have an uneven number of slides, use a HybriSlip Hybridization coverslip for the final slide. If you are not using ProbeOn Plus slides with which you can make 'slide sandwiches' because of the raised tabs, you will treat each slide individually and use flexible coverslips, instead.

△ **CRITICAL:** Handle formamide in a fume hood.

139. When the probes are thawed, dilute the hybridization probes with formamide.
 - a. Mix equal volumes of the different probes for a given gene of interest (i.e., 5'-UTR antisense probe + central antisense probe + 3' antisense probe; [Figure 2F](#)).
 - b. For a 1× concentrated probe mix: Add 1 μL of RNA probe mix to 50 μL 50% formamide in RNase-free water for 1 slide pair.
140. Denature the diluted probes for 3 min at 98°C. Immediately, place them on ice. Further dilute hybridization probes with hybridization solution.
 - a. For 1 slide pair: Add 200 μL of hybridization solution.
141. Pipette 220 μL of prepared hybridization probe on one slide of a slide pair
142. Lay one slide onto the other, sections facing each other, and make sure that no bubbles remain. Place the slide sandwich on the rubber-lined risers in the hybridization chamber. Avoid touching the slides, which could cause cross-contamination. It is also recommended to change the gloves between samples with different probes.

Alternatives: If slide sandwiches are not feasible, add 200 μL of hybridization probe on a SuperFrost Ultra Plus GOLD Adhesion slide and cover it with a HybriSlip Hybridization coverslip.

143. Close the lid of the hybridization chamber and seal with parafilm.
144. Carefully place the chamber in the oven at 55°C.
145. Place a beaker filled with 500 mL H₂O in the oven for 12 to 14 h to maintain humidity, as well as the prepared 0.2× SSC to pre-warm it.

Washing

146. Set incubator to 55°C.
147. 0.2× SSC can be microwaved to reach 55°C if not pre-warmed.
148. Carefully, remove hybridization chamber from the oven.
149. Separate the slide pairs by dipping them into pre-warmed 0.2× SSC solution.

Alternatives: If slide sandwiches are not feasible, remove the coverslip by dipping the slide into pre-warmed 0.2× SSC solution.

150. Place separated slides into the slide rack.
151. Place the slide rack into the slide container and fill it with pre-warmed 0.2× SSC solution.

Note: Be careful not to pour SSC directly on the sections, but on the labeling end of the slide. Alternatively, remove the slides, add the SSC and replace the slides.

152. Place the slide container in the incubator and gently shake at 55°C for 1 h.
153. Replace 0.2× SSC solution and incubate gently shaking at 55°C for 1 h.
154. Again, replace 0.2× SSC solution and incubate gently shaking at 55°C for 1 h.

Blocking

155. Wash slides in 1× PBS on an orbital shaker for 5 min at 22°C ± 2°C.
156. Move slides into a plastic box, where slides can be arranged facing up.
157. Incubate slides with 50 mL block solution 1 on an orbital shaker for 45 min at 22°C ± 2°C.
 - a. Ensure that the slides do not slide over each other to avoid damaging the sections.
158. Remove block solution 1 from the slides using a serological pipette.
159. Incubate slides with 50 mL BSA block solution 2 on an orbital shaker for 45 min at 22°C ± 2°C.

Antibody

160. Prepare the antibody solution before the incubation time is over. Dilute anti-Dig alkaline phosphatase conjugated antibody (see [key resource table](#)) 1:1250 in BSA block solution 2. Mix 4 μL antibody with 5 mL BSA block solution 2.
161. Remove slides from the plastic box by arranging them on a fresh paper towel.
162. Place the slide pairs next to each other and fill the hybridization chamber with 200 mL H₂O.
163. Pour the antibody dilution into a weigh boat and treat slides with antibody dilution ([Figure 3](#)):
 - a. Make slide sandwiches with respective slide pairs with sections facing towards each other.
 - b. Place the short edge of the slide sandwich on a stack of paper towels to wick out any solution.
 - c. If all liquid is wicked away, dip the slide sandwich edge into the antibody solution to wick it away until all sections are covered.
 - d. Press the slide sandwich edge to the paper towels to wick away the antibody dilution, and then dip slide sandwich edge into the antibody solution again, until all sections are covered and avoid bubbles.
 - e. Dry the outside of the slide sandwich with a paper towel and place it in the hybridization chamber.
 - f. Close the lid and seal it with Parafilm.

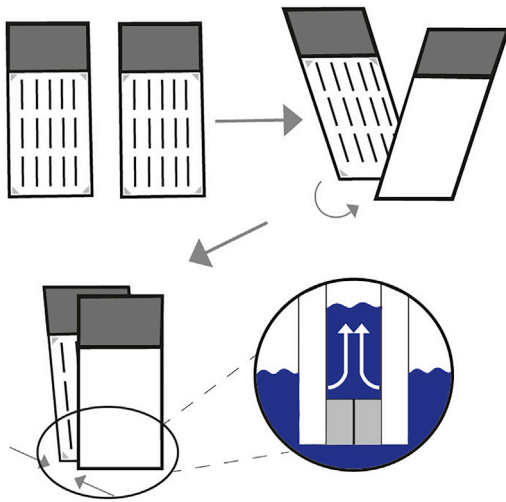


Figure 3. Handling of slide sandwiches

Move the bottoms of two slides in the treatment solution against each other. Carefully bring the rest of the slides together until the tops are touching. The sections will be protected by spacers on ProbeOn Plus slides. The treatment solution will be wicked away by capillary forces.

g. Incubate for 2 h at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$.

Alternatives: If slide sandwiches are not feasible, add 200 μL of antibody dilution on a Super-Frost Ultra Plus GOLD Adhesion slide and cover it with a HybriSlip Hybridization coverslip.

Washing

164. Move the slides out of the hybridization chamber and separate them carefully.
165. Place single slides back into the plastic box to lay them tissue facing up. Place the box on an orbital shaker.
166. Wash slides with 50 mL BSA blocking solution 2 for 15 min at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$.
167. Wash slides with 50 mL BSA blocking solution 2 for 15 min at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$.
168. Wash slides with 50 mL BSA blocking solution 2 for 15 min at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$.
169. Wash slides with 50 mL BSA blocking solution 2 for 15 min at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$.
170. Wash slides with 50 mL buffer C for 15 min at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$.

Detection

Note: Though there are several possible detection methods for *in situ* hybridization, we find that the reaction of alkaline phosphatase with BCIP/NBT (see [key resource table](#)) gives a reproducible result for various genes on maize leaf sections. The detection reagent BCIP is hydrolyzed by alkaline phosphatase conjugated to the secondary antibody to form an intermediate leucoindigo compound, which is oxidized by NBT to form 5, 5'-dibromo-4,4'-dichloro indigo (BCI). NBT is reduced to insoluble purple diformazan (DF) and forms NBT-DF. The combination of NBT-DF and BCI results in a dark purple colored precipitate. The color of the stain may vary from blue to purple to brown.

171. Remove slides from the plastic box. Arrange pairs next to each other on a fresh paper towel.
172. Fill the hybridization chamber with 200 mL H_2O .
173. Add 50 μL NBT and 37.5 μL BCIP to 10 mL buffer C.
174. Pour the solution into a weigh boat and keep protected from bright light.
175. Wick NBT-BCIP detection solution into the slide sandwiches as described for antibody treatment ([Figure 3](#)).
176. Place them in the hybridization chamber, close the lid and seal it with Parafilm.
177. Incubate slides with detection solution for several hours to 3 days.

- a. The duration depends on several factors such as abundance of mRNA and probe quality, but the staining process can be checked periodically.
 - b. Typically, *in situ* hybridization results are detectable as a purple precipitate after one night.
178. After checking the staining progress of one slide, apply fresh detection solution if staining has not reached the desired intensity or after 24 h of staining.
179. Stop further incubation when purple precipitate can be observed but background noise is still low.

Note: The detection step is light sensitive. Keep light exposure times as short as possible.

Note: To stop the detection, an ethanol series is performed to reduce background staining. Notice that the stain is also slightly ethanol soluble and longer incubation periods will reduce the intensity of staining. This is particularly important for very faint signals, as the signal can be washed away by the ethanol steps.

Stop detection

- 180. Separate slide sandwiches by carefully placing them in water, allowing them to fall apart.
- 181. Place separated slides into the slide rack.
- 182. Incubate in water for 3 min.
- 183. Incubate in 30% ethanol for 30 s.
- 184. Incubate in 50% ethanol for 30 s.
- 185. Incubate in 70% ethanol for 30 s.
- 186. Incubate in 80% ethanol for 30 s.
- 187. Incubate in 90% ethanol for 30 s.
- 188. Incubate in 100% ethanol for 30 s.
- 189. Incubate in 100% ethanol for 30 s.
- 190. Incubate in Histo-Clear for 5 min.
- 191. Incubate in Histo-Clear for 5 min.

△ CRITICAL: To mount the slides, work under a fume hood and let your slides dry there, as well.

Mounting

- 192. Move slides on a fresh paper towel. [Troubleshooting 5](#)
- 193. Add a line of 120 μ L Eukitt on the slide and cover it with a coverslip. Avoid bubbles.
- 194. Let the mounting medium solidify for 12 to 14 h.

Imaging

⌚ **Timing:** 1 h

In this section the slides will be observed under a light microscope. Purple precipitate will locate the mRNA of the gene of interest.

Microscopy

- 195. Observe sections on a standard bright-field microscope. [Troubleshooting 2, 3, and 4](#)
 - a. For bundle sheath cells of maize plants, a 20 \times magnification is optimal.

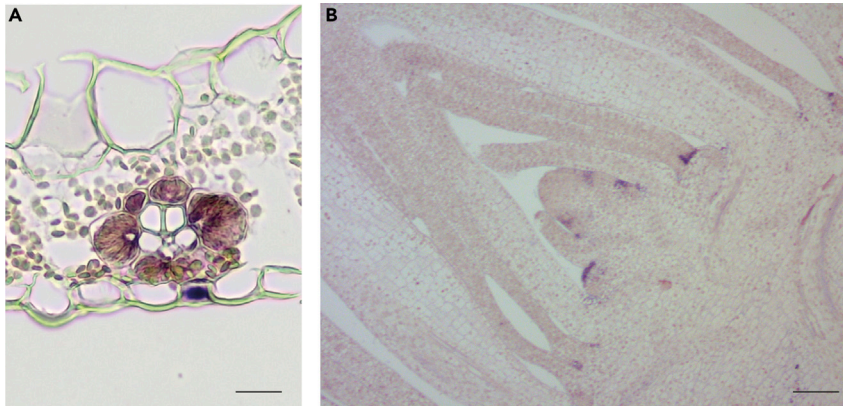


Figure 4. Expected outcome

(A) *In situ* hybridization of *ME1*. Rank-2 intermediate vein from leaf section hybridized with antisense probe shows mRNA localization specifically to bundle sheath cells. Scale bar, 50 μm .

(B) *In situ* hybridization of *CUP-SHAPED COTYLEDON2 (CUC2)* in the maize shoot apical meristem. *CUC2* marks the organ boundaries and specific expression is observed at the edges of the leaf primordium and at the boundaries between stem and leaves. Scale bar, 100 μm .

EXPECTED OUTCOMES

Sections hybridized with an antisense probe should show purple stain in the region or cell type where the mRNAs of interest are located. Sections treated with sense probes should not have any stain. NBT-BCIP incubation of corresponding sense and antisense probes should be stopped together to maintain comparable conditions (Figures 4A and 4B).

LIMITATIONS

The protocol can be applied to detection of mRNAs in monocot leaves and other tissues with similar properties. The use of Paraplast as embedding medium is optimal for mature leaves but may not be suitable for tissues which are softer, such as *Arabidopsis* leaves, or harder, such as maize kernels. In this case, another embedding medium such as LR white resin may be preferable.

TROUBLESHOOTING

Problem 1

Paraffin ribbons rip during sectioning e.g., during microtome sectioning.

Potential solution 1

1. Change the blade angle. We usually position the blade at an angle of 7.5 degrees relative to the tissue.
2. If the room temperature is too hot or too cold, it can affect the consistency of the wax. If possible, keep it at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$.
3. If the sample has been at 22°C , place it back on ice and let it cool again.
4. Wipe the edges of the section with a wet brush, occasionally.
5. Use a fresh blade.
6. Trim the sample with a razor blade back to a trapezoidal shape.
7. Rearrange the sample to the blade. It should be parallel.
8. Pause and come back when a calm state of mental clarity is reached.
9. Tissue is not suitable for paraffin embedding.

Problem 2

No stain can be detected while imaging.

Potential solution 2

1. Use a positive control probe, for example, in leaf tissue: RuBisCO small chain homologs (RBCS1 or RBCS2) have high levels of mRNA in bundle sheath cells. In the shoot apical meristem working probes have been published for a multitude of genes such *CUP-SHAPED COTYLEDON2* (Johnston et al., 2014) (Figure 4B).
2. RNA is degraded. Perform *in situ* hybridization steps absolutely RNase-free.
3. Antibody detection incubation time is too short. Incubate in NBT/BCIP longer.
4. Permeabilization step was too long.
5. Permeabilization was not successful. It is crucial for sample penetration.
6. NBT/BCIP has expired. Use fresh solutions and reduce light exposure to a minimum.
7. Ethanol de-staining washes were too intensive and reduced the stain intensity accordingly. Reduce ethanol incubation time.
8. Probe did not bind to transcripts. Make new probes in a different region.

Problem 3

Paraffin sections under the microscope are not intact or have disintegrated.

Potential solution 3

1. The tissue was not completely fixed. Be careful that the tissue sinks after pulling a vacuum. If FAA was used instead of PFA the incubation time has to be adjusted. In our experience 1 h fixation in FAA is insufficient.
2. We highly recommend ProbeOn Plus Slides (see [key resource table](#)). They feature small spacers so that you can make slide sandwiches without removing the samples. If these are not available (as in Europe), it is not possible to make slide sandwiches and these steps must be performed with flexible coverslips.
3. Replace the blade of the microtome. If the blade is blunted or notched it can damage the tissue.

Problem 4

During imaging, staining is detected in cells above and below major and/or rank-1 intermediate veins of maize leaves, even in sense probe-hybridized sections.

Potential solution 4

These are hypodermal sclerenchyma cells, and staining of these cells is considered unspecific since also seen when using sense control probes in maize leaves.

Problem 5

I want to use a counterstain to see cell walls more clearly e.g., when mounting.

Potential solution 5

In our experience, counterstaining is not necessary to visualize cell walls. However, several stains are compatible with this protocol. Classical counterstains such as Eosin-Y require xylene-containing mounting media, such as Eukitt, for visualization. Though NBT/BCIP may form crystals when mounted with xylene-containing media, we have not encountered this problem. In principle, Eukitt mounting medium and Eosin-Y counterstain are compatible with this protocol. As an alternative, Xylene-free mounting media such as Vectamount can be used instead, with a compatible counterstain such as Vector Methyl Green or Vector Nuclear Fast Red.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Dr. Wolf B. Frommer (frommew@hhu.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

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AUTHOR CONTRIBUTIONS

Conceptualization, N.R.Z., M.B., and R.L.; methodology and investigation, N.R.Z., M.B., and R.L.; resources and supervision, H.N., W.B.F., and R.S.; and writing, N.R.Z., M.B., and W.B.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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