Supplementary Information for

Sensitive whole mount *in situ* localization of small RNAs in plants

by

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Detailed Protocol for whole mount in situ hybridization of small RNAs:

i) Fixation and Dehydration

All the steps are carried out at room temperature unless mentioned otherwise.

Details on the preparation of the solutions underlined are indicated at the end of the protocol.

1) Put whole seedlings in 4% PFA (in PBS), for 45mins with vacuum infiltration. Embryos were dissected in PBS, then immediately transferred to baskets immersed in 4% PFA in PBST (PBS + 0.1% Tween) on ice. Embryos were fixed for 1-3 hours with vacuum infiltration at room temperature and then washed three times for minutes each in PBS.

2) When using slides (use high adhesion slides such as Menzel SuperFrost Ultra Plus®) before starting set up a dry heating block at 42°C. Put 20-25 seedlings on glass slides (one slide per probe) ; add a coverslip and apply very gentle pressure (without damaging the seedlings) ; snap freeze in liquid nitrogen; remove coverslip immediately and dry at 42°C heating block (do not over dry).

   When using liquid handling robot (e.g. INTAVIS *In-situ* Pro vs) , transfer seedlings in small baskets and place the baskets in 96-well plate of the liquid handling robot.

3) Incubate two times for 5mins each in methanol and two times for 5mins each in ethanol (absolute). Storage at this point is possible.

4) Prepare a 1:1 solution of absolute ethanol and Histo-Clear® and permeabilize samples by incubation for 30mins in the solution.

5) Wash twice for 5mins in 100% ethanol (Note: Prepare humidified chamber if glass slides used, square plastic petri plates with folded tissue soaked in soaking solution at the bottom).

6) Rehydrate in 95%, 90%, 80%, 60% ethanol/NaCl and 30% ethanol/NaCl solutions for 2mins each. Embryos were rehydrated in 90%, 70%, 50% and 30% ethanol for 10 minutes each,

then for 2 minutes in Proteinase K buffer.

7) Incubate in 0.75% NaCl for 2mins, then rinse in PBS for 2mins.

PAUSE POINT: At this step slides or baskets can be left for several hours in PBS buffer in a sealed container.

ii) Pretreatment

8) Incubate with 37°C preheated Proteinase K solution for 30mins. If using slides, add 100μl when using slide and cover with a sheet of plastic. For baskets use an appropriate amount to submerge the samples. Embryos were incubated in proteinase K (75μg/ml in Proteinase K buffer) at 37°C for 15 mins.

9) Neutralize the Proteinase K with glycine (2 mg/ml) in PBS for 5mins.

10) Rinse for 5mins in PBS or PBST for embryos.

11) Post-fix in 4% PFA (in PBS) for 5mins or PFA in PBST for embryos.

12) Wash twice for 5mins each in PBS or PBST for embryos.

13) EDC treatment [to fix small RNAs] (optional step).

14) Incubate two times in methylimidazole-NaCl for 10mins each.

15) Incubate for 2hrs in EDC cross link solution at 60°C.

16) Stop the reaction with glycine (2mg/ml) in PBS for 5mins.

17) Wash twice for 5mins in PBS+0.1% Tween. After three 5 minute washes, embryos were washed once in hybridization mix (without probe) for 5 min. For embryos skip to step 21 directly.

18) Incubate 30min ethanolamine (triethanolamine) -acetic anhydride solution. This step was not performed for embryos.

19) Rinse in PBS for 5mins.

20) Incubate in 0.75% NaCl for 2mins, afterwards, dry as much as possible.

iii) Hybridization and Wash

21) Denature LNA probe in Hyb Mix (hybridization mixture) for 2mins at 80°C.

Critical step: The LNA probe concentration should be determined by testing a series of dilutions. For each of the probe that we used the concentrations are mentioned in Table 1. For embryos each probe was used in a final concertation of 20nM.

22) Preheat 2xSSC and 0.2xSSC buffers to 65°C for washing steps the next day

23) When using slides add 100μl probe onto slide and seal with plastic coverslip or, when using baskets, the appropriate amount to submerge the samples. Incubate in humidified chamber at 65°C or 55°C (depending on the probe) o/n (16-20 hrs). Put the slides in a humidified box and seal the box tightly with paraffin film. Embryos were immersed in 800 μl hybridization mix with

probe in 24-well plates, which were placed in a humidified chamber (Tupperware box filled with wet tissue paper) and incubated at 65°C for 16 hours.

Critical step: At this step care should be taken when using slides after adding the probe there are no air bubbles in between the slide and the coverslip. Typically the hybridization temperature ranges between 55° and 65°C. We used 65°C for embryos.

24) Remove coverslips carefully and wash in 2xSSC for 2min at 65°C.

25) Wash in 0.2xSSC two times for 30mins each at 65°C or at the same temperature as used for the hybridization.

26) Wash in 0.2xSSC for 5mins at 37°C (skip this step for embryos).

27) Wash in 0.2xSSC for 5mins at room temperature (skip this step for embryos).

28) Wash for 2mins in PBS.

29) Block in PBS-Tween 1% BSA for 30mins at room temperature. Make sure while using slides that the blocking buffer uniformly covers all over the samples to ensure effective and uniform blocking.

30) Incubate with anti-Digoxigenin (1:1250; in PBS-Tween, 1% BSA) for 90mins at room temperature (use plastic coverslips for slides). Ensure uniform treatment of the antibody mixture all over the samples.

31) Remove coverslips, wash 2x30mins in PBS-Tween + 1% BSA at room temperature. After antibody reaction embryos were washed once in a blocking solution then 2x10 mins in PBS-Tween.

32) Wash 30mins in PBS-Tween at room temperature.

33) Wash twice with TNM-50 for 5mins each.

34) Prepare NBT-BCIP solution (1:50 in TNM-50, should be fresh) , apply on slides (glass coverslips).

35) Place slides in humidified box (water) and incubate at RT in the dark.

Monitor for the appearance of the signal regularly. Revelation ranges from 10 minutes to overnight.

Critical step: Keep monitoring for detection of signals at regular intervals.

36) When signal can be detected remove coverslips when using slides and stop reaction with 1X TE-buffer by washing three times for 5mins each. When using baskets wash with 1X TE-buffer by washing three times for 5mins each.

Critical step: Be careful if using slides, so that the tissue does not wash away to much at this step.

37) Transfer samples from baskets to slides. Embryos were carefully transferred to glass slides with 3 wells, 11.28mm well diameter slides (Electron Microscopy Science Cat. No. 63418-11). TE buffer was exchanged to 70% glycerol in TE and a long (24x50 mm) cover slip was gently applied to cover embryos. Slides were sealed with nail polish and stored at 4°C.

iv) Microscopy

For imaging a light microscope equipped with Nomarski (Differential Interference Contrast, DIC) and a 20X objective lens is used. Embryos were scanned in Pannoramic SCAN 150: an automated widefield microscope equipped with WhiteLight LED for transmitted light, objective 20/0.8 plan-apochromat, and the camera Hitachi HVF22 – transmitted light. Images were captured using the Pannoramic Viewer software.

General recommendations: The key step is the optimization of probe hybridization temperatures for each of the probes used. The optimal temperature may vary based on the specific LNA oligo probe being used. A range of temperatures can be used between 55° to 65°C. The probe concentration should be tested. We have used 10nM concentrations. Very importantly, control should be used. Here we used two types of controls; either a control probe in sense orientation to the miR390 as a negative control and the mouse microRNA miR124. A control without any LNA probe added can also be performed.

Reagents and solutions:

-4%PFA (Paraformaldehyde) (Sigma Aldrich-16005) in PBS (for 100ml): Add 4g of PFA in deionized water and add 1M NaOH; stir and heat at 65°C until PFA is dissolved; add 10ml 10X PBS buffer and allow it to cool down to RT; adjust pH to 7.4 using 1M HCL and adjust volume to 100ml.

-1x PBS

-Methanol

-Ethanol

-Ethanol & Histo-Clear® (Roti®-Histol from Roth; Art.no.6640.1): 1:1 (v/v)

-Soaking solution: formamide 17.5ml

20xSSC 3.5ml

Water 14ml

-Ethanol series:

Ethanol series	EtOH(ml)	Water(ml)	NaCl 5M(ml)
95% EtOH	95	5	
90% EtOH	90	10	
80% EtOH	80	20	
60% EtOH +NaCl	60	36	3
30% EtOH +NaCl	30	68	3

-0.75% NaCl (w/v)

-Proteinase K (Recombinant PCR grade from Roche; REF 03115828001):

final concentration 125µg/ml (10µl of a 20mg/ml stock in 1.6ml Proteinase K buffer)

Proteinase K buffer (for 200ml): 1M Tris (pH-7.5) 100ml

0.5M EDTA 99.5ml

Acetic anhydride 0.5ml

-Glycine (2 mg/ml) in PBS

-Methylimidazole-NaCl (1 Methylimidazole; 336092 Sigma Aldrich)

-13M methylimidazole 10.362ml/L

-300mM NaCl 75ml 5M NaCl stock/L

-pH 8.0/HCl

-EDC: 0.16M in methylimidazole-NaCl 31.632g/L

(N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (Sigma E7750; stored at -20°C)

Ethanolamine (triethanolamine) -acetic anhydride solution (100ml)

-Ethanolamine 0.1M

-Acetic anhydride 0.5%

-Water up to 100ml

-PBS+0.1% Tween: 20µl in 20ml

-HybMix buffer: deionized formamide 5ml

-50% dextrane sulfate 2ml

(Dextran sulfate sodium salt from *Leuconostoc spp.*-Sigma-D-7037)

-10x in situ salts 1ml

-50x denhardt reagent 0.2ml

-tRNA 50mg/ml 0.1ml

-Water 1.7ml

-2xSSC (Dilute from 20x SSC)

-0.2xSSC(Dilute from 2x SSC)

-PBS-Tween + 1% BSA: 0.4g BSA in 40ml PBS-T

-NBT-BCIP(Roche; REF-11681451001) (1:50; in TNM-50): 32µl in 1.6ml

-TNM-50 (for 200 ml): 1M Tris pH9.5- 100ml

5M NaCl - 20ml

1M $MgCl_2$- 50ml

Water - 30ml

-1xTE (Dilute from 10x TE)

-TE/glycerol: 1:1

-Anti-Digoxigenin-AP (Fab fragments from sheep from Roche: cat # 11093274910 200 µl 150U)

Stocks solutions

• **5M NaCl 1L**	NaCl	292g
	H_2O	1L
• **10xPBS 1L**	NaCl	76g
	NaH_2PO_4	4.14g
	Na_2HPO_4	25.07g
	pH7.0 w/HCL	
	H_2O	Up to 1L
• **20xSSC 1L**	NaCl	175.3g
	Na-citrate	88.2g
	pH 7.0/HCl	
	H_2O	Up to 1L
• **Na-phosphate 100ml**	1M Na_2HPO_4	46.3ml

	1M NaH_2PO_4 pH6.8	53.7ml
	5M NaCl	6ml

- **10x in situ salts 10ml**

	1M Tris pH8	1ml
	1M Na-phosphate pH6.8	1ml
	0.5M EDTA	1ml
	H_2O	Up to 10ml

- **50x denhardt's 10ml**

	Ficoll 400	0.1g
	polyvinylpyrrolidone	0.1g
	BSA	0.1g
	H_2O	Up to 10ml

10xTE 1L

	1M Tris	100ml
	0.5M EDTA	20ml
	H_2O	Up to 1L