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In situ PCR using leaf epidermal peels

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Manuscript citation:
Pornsiriwong, W. et al. (2017). A chloroplast retrograde signal, 3′-phosphoadenosine 5′-phosphate, acts as a secondary messenger in abscisic acid signaling in stomatal closure and germination. Elife 6: 1–34.

Protocol status: Working
We have used this protocol and it worked.

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Abstract

Method for visualizing cell-localized gene expression patterns.

Adapted from:
Athman, A., Tanz, S.K., Conn, V.M., Jordans, C., Mayo, G.M., Ng, W.W., Burton, R.A., Conn, S.J., and Gilliham, M. (2014). Protocol: a fast and simple in situ PCR method for localising gene expression in plant tissue. Plant Methods 10: 29.

Also, for more information on epidermal peels:
Chen, Z.-H., Eisenach, C., Xu, X.-Q., Hills, A., and Blatt, M.R. (2012). Protocol: optimised electrophyiological analysis of intact guard cells from Arabidopsis. Plant Methods 8: 15.

Guidelines

Remember that general RNA guidelines apply. Make sure to clean benches and fume hood area with ethanol/RNAzap before performing experiment to minimize chances of contamination. All solutions should be RNAse free and use filter tips. Do not let slides dry out at all. Do not use a centrifuge.

You will require a PCR machine with a heating block that accepts microscope slides (e.g. GSTORM1 thermal cycler).

Materials

MATERIALS

- Formaldehyde
- Sodium phosphate dibasic **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**7558-79-4
- Ethanol
- scalpel blades
- NaCl
- Turbo DNA-free Kit **Invitrogen - Thermo Fisher Catalog #**AM1907
- BSA
- Microscope slides
- 0.5M EDTA, pH 8.0 **Thomas Scientific Catalog #**C837L97
- Razor blade
- Tris-HCl **Life Technologies Catalog #**AM9855
- Thermal cycler
- glacial acetic acid
- fine paint brush
- Thermoscript Reverse transcriptase **Invitrogen - Thermo Fisher Catalog #**12236
- acetic acid
- clear nail polish
- DIG-11-dUTP
- Anti-DIG
- Frame seal **Bio-Rad Laboratories**

Safety warnings

Take care with scalpel/razor blades, tweezers and formaldehyde (in fixative; wear gloves and use fume hood).

Before start

Make sure you are comfortable with performing leaf peels to study epidermal cells (visualize in opening buffer and measuring buffer under light microscope and see responsive stomates).

The day before: prepare slides by placing frame-seals on glass slides - apply reasonable pressure on borders to seal. Can heat in thermal cycler at 90C for 10min. Cut off excess seal to allow slide to sit appropriately in PCR machine.
Make sure to make fresh fixation solution each time.

Slide preparation (day before)

1 **The day before**: prepare slides by placing frame seals on glass slide and apply pressure to borders to seal. These can be heated in thermal cycler at 90 °C for 10 minutes. Cut off excess seal to allow slide to sit appropriately in PCR machine.

Sample preparation and fixation

2 Prepare fresh fixative and keep on ice:
- Fixative: 63% Ethanol, 5% acetic acid. 2% formaldehyde.

Prepare fresh and use within 3 hours.

3 Bring plants to fume hood for epidermal peeling along with fixative, microscope slide and frame-seal. Keep all on ice for fixing and washing peels.

4 Add 100ul fixative onto all microscope slides.

5 Make epidermal peels (try to place 3-5 moderately sized peels per slide) and place directly into fixative on slide using a fine paint brush. Randomise which peels go to which slide (at this point, there should be no allocation of which slide will be used for which gene – can make this decision based on peel + slide quality).

Wear gloves, handle formaldehyde with care.

6 Incubate peels in fixative for 45 minutes, refreshing the fixative (with pipette) approximately every 15 minutes (apply evenly across peels on each slide).

Washes

7 1. Wash 3 × 10 minutes in 100 µl ice-cold ***Wash Buffer 1*** (63% Ethanol, 5% glacial acetic acid. 4°C).

8 After last wash, remove as much wash buffer as possible.

9 Wash 3 × 5 min in 1x PBS (0.1 M Na_2HPO_4, 1.3 M NaCl, pH 7.5, stored at room temperature <1 year).

10 From this point, the remainder of the protocol can be performed at lab bench. Wipe off any excess liquid to ensure no formaldehyde remains.

SAFE PAUSE POINT

DNAse treatment

11 Setup Turbo DNAse (Ambion) mastermix.

	Component	x1 rxn (µl)
	sterile milliQ H20	87
	10X Buffer	10
	DNase	3

DNAse mastermix recipe

12 Remove PBS solution and add 100 µl of mastermix to each slide.

13 Incubate 37 °C for 45 mins.

14 Add 3 µl 0.5 M EDTA to give final concentration of 15 mM.

15 Heat inactivate at 70 °C for 15 mins. Place on ice.

NOTE: Do not cool in thermal cycler as it can lead to cracking of glass slides.

16 Remove DNase mix and wash 2x with ice-cold sterilised water.

Do not remove final wash before RT reaction mix is ready!

17 At this point, it must be decided which genes are being studied on which slides. There must be two control slides: one positive housekeeper (e.g. 18S rRNA) control and one no RT housekeeper negative control.

Reverse transcription

18 Setup RT mix (Thermoscript RT or Superscript III)

	Component	x1 rxn (µl)

	Sterile milliQ H20	61
	5X Buffer	20
	10 mM dNTPs	10
	Reverse primer (20 uM)	2.5
	DTT 0.1 M	5

Thermoscript RT recipe

19 Add 98.5 µl of mastermix to all slides

20 Incubate slides at 65 °C for 5 minutes, then place on ice for 1 min.

21 Add 1.5 µl of RT enzyme (Thermoscript or Superscript III) to all slides EXCEPT negative housekeeper control. Pipette up-and-down gently to mix around.

22 Run thermal cycle at 55 °C for 30 mins, 70 °C for 15 mins, then hold at 4°C (better to take out of machine and keep on ice).

This is a safe PAUSE POINT (keep slides covered in fridge).

In situ PCR

23 Remove RT solution from slides (keep slides on ice).

24 Wash slides twice for 1 min with 100 µl ice-cold sterile water. When ready to proceed, remove as much water as possible (use a 10/20 µl pipette to get as much as possible).

25 Setup PCR mix (HiFi Phusion Taq)

	Component	per slide (µl)
	Sterile milliQ H20	74.6
	5X Buffer	20
	10 mM dNTPs	2
	polymerase	0.5
	DIG-11-dUTP (1 mM)	0.4
	Reverse primer (20 µM)	1.25
	Forward primer (20 µM)	1.25

HiFi Phusion Taq PCR Mix

26 Distribute PCR mix equally across the slide by pipetting and run PCR:

1. 95 °C 30 min
2. 95 °C 10 min
3. 60 °C 25 min
4. 72 °C 5 min
5. Return to step 2 20–35x
6. 72 °C 7 minutes
7. 10 °C hold (better to take slide out).

Colorimetric detection of DIG labelled products

27 Remove frame-seal chamber completely and perform remainder directly on slide. During incubation, place slide in small plastic box to minimise evaporation.

28 Pipette off remaining PCR solution.

29 Wash twice for 5 min in 100 µl 1x PBS.

30 Gently add 100 µl of 1x *Block solution* and incubate 30 min on ice.

10X Block solution: 10 mg BSA in 1 mL 10x PBS

31 Dilute anti-DIG-AP antibody 1:500 in 1x Block solution, keep on ice.

32 Pipette off as much 1X block solution as possible.

33 Add 50 µl of diluted anti-DIG-AP antibody and incubate at room temperature for 1 hour.

34 Remove antibody solution and wash twice for 15 min at room temperature in *Wash Buffer 2* (can transfer peels to another microscope slide if desired; keep in wash buffer 2).

Wash Buffer 2: 0.1 M TRIS-HCl, 0.15 M NaCl, pH 9.5

35 Remove wash buffer 2.

36 Add 50 µl BM purple (AP substrate). This is light sensitive, incubate in dark (e.g. drawer, or cover) and keep on ice.

37 Staining can occur anywhere between 10 min – 2 hours. Optimize as appropriate. If no signal is detected after 1 hour, refresh BM purple (take off and add another 50 µl).

Microscopy

38 Once stain has developed, pipette off BM purple.

39 Add 100 µl Wash Buffer 2 and wash 3× 5 min to remove residual stain and debris.

40 Wash once with sterile water, then remove all water.

41 Mount section in 40 µl 40% glycerol (40–150 µl depending on number of peels on slide). Do not use excessive amounts of glycerol.

42 Place coverslip on top and seal corners with nail varnish. Coverslip should not float (remove some glycerol if needed). **NB** do not slide coverslip, instead just drop directly onto peels/glycerol.

43 Visualise under bright field microscope. Slides can be stored at room temp and is good ~3 weeks. Ensure all peels are imaged using the same settings.