SCHNABLE LAB
Plant Genomics

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FIXATION, DEHYDRATION, PARAPLAST INFILTRATION AND SECTIONING PROTOCOL WITH TISSUE COLLECTION PROCEDURE USING THE PALM LASER MICRODISSECTION SYSTEM

(Last Revised: October, 2007)

The following protocol for maize shoot apical meristems (SAMs) was adapted by Dr. Kazuhiro Ohtsu and Marianne Smith of the Schnable Laboratory (Iowa State University) from several sources: Kerk et al., Plant Physiol (2003), 132, 27-35; Asano et al., Plant J (2002), 32, 401-408, and from own laboratory notes; using support from a grant from the National Science Foundation (DBI0321595). Please contact Dr. Patrick Schnable (schnable@iastate.edu) regarding questions or corrections.

The citation for this protocol is:

Ohtsu K, M Smith, SJ Emrich, LA Borsuk, R Zhou, T Chen, X Zhang, M Timmermans, J Beck, B Buckner, D Janick-Buckner, D Nettleton, MJ Scanlon, PS Schnable (2007) **Global gene expression analysis of the shoot apical meristem of maize (Zea Mays L.)**. The Plant Journal 52: 391-404.

Fixative

100% Acetone

Fixation

Day one

1. Pure ice-cold acetone was used as fixative. Gloves were worn at all times. The procedure is to be carried out in a hood.
2. Seedlings were brought into the lab shortly before fixation. Seedlings were cut off at the coleoptile node with a new single-sided razor blade and immediately placed in a glass Petri dish containing ice-cold acetone. A second cut, approximately one cm above the coleoptile node, was executed immediately.
3. Quickly the sides of the tissue block were trimmed until a square block of approximately 0.3 x 0.3 x 0.2 cm was obtained, always keeping the sample submersed in the fixative.
4. The resulting tissue block was placed in a scintillation vial with 15 ml ice-cold fixative (100% acetone) and kept on ice.
5. Eight to ten seedlings were prepared in the same way and added to the same vial. Total preparation time of 8-10 seedlings per vial should be kept around 5-10 minutes.
6. A small plastic container holding ice and vial were submitted to a vacuum of 400mm Hg for 10-15 minutes and then slowly brought back to room pressure.
7. After vacuum infiltration the fixative was replaced by fresh ice-cold acetone, and the vial was placed on a rotator for 1 hr at 4°C.

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8. The tissue was then emptied into a Petri dish, placed under a dissecting microscope, and extra leaf and nodal tissue was removed using a scalpel. This results in approximately 0.3 x 0.2 x 0.1 cm tissue blocks.
9. The vacuum infiltration procedure (6 and 7) was repeated twice (in total, 3 infiltrations). Samples remained in the last change of fixative over night in a 4 ° environment on a rotator.

Dehydration/Xylene Infiltration
Day two

10. The following morning the samples were brought to room temperature (RT) and given a fresh change of acetone at RT and then placed on a rotator at RT for 1 hr.
11. After that time lapse the fixative was replaced by a mixture of 1:1 acetone : xylene, and placed on a rotator at RT for 1.5 hrs.
12. If the target tissue is very dense, or hidden under various other tissue layers, gradual infiltration with xylene may be done slower, using 3:1, 1:1, and 1:3 mixtures of acetone : xylene.
13. Acetone replacement was followed by three changes of pure xylene for approximately 1hr each.

Paraplast Infiltration

14. A small amount of paraplast chips were placed into vials after the third pure xylene change and left to dissolve overnight (o.n.) on a rotator at RT.

Day three

15. Next morning the vials were placed in a 60 °C warm oven to melt any chips not yet dissolved. When paraplast was melted, vials were gently inverted back and forth to mix xylene and Paraplast evenly.
16. After 1.5 hrs more Paraplast chips were added for melting and left for an additional 1.5 hrs.
17. Half of this mixture was discarded and replaced with pure molten paraffin, and mixed by inversion and left o.n. in the 60°C warm oven.

Day four

18. Next, all of the mixture was replaced with pure Paraplast. Throughout day four a total of at least three pure Paraplast exchanges were done, each lasting at least 4 hours.

Day five

19. Exchange Paraplast early in the day, leave another 4 hrs at least, and embed.

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Embedding

20. Tissues were embedded using Tissue Tek base molds (metal) together with Tissue Tek embedding rings.
21. The embedding procedure was carried out using a "home-made" device consisting of a gradient metal warming plate (hot on one end and cold [RT] on the opposite end). Paraplast was dispensed from a 2.5 L reservoir into the assembled base molds plus embedding rings.
22. Tissue and Paraplast were poured from the vial into a metal weighing dish on the hot side of the warming plate. The tissue was scooped out with a weighing spatula, placed into the assembled base mold/ embedding ring combination, and oriented for sectioning on a rotary microtome.
23. Blocks were first cooled down to RT and then placed on ice for easy un-molding.
24. Blocks were kept in plastic bags at 4°C.

Sectioning

25. Paraplast blocks were trimmed into a narrow trapezoidal shape with parallel horizontal (10 µm thick) cuts at top and bottom, and slanted cuts on the sides. The blocks were sectioned on a Leica RM 2135 rotary microtome.
26. Ribboned sections were placed on Fisher probe-on+ slides, and floated on diethyl-pyrocarbonate (DEPC) treated water at 40°C on a slide warming tray for approximately 5 minutes, or until the sections stretched (not to exceed 20 minutes).
27. Water was removed by tipping the slide onto absorptive paper towels while holding one end of the ribbon with a fine paintbrush. Residual water was wicked off with tissue paper. Slide should not cool down during the water removal, and afterwards must be quickly placed back on the slide-warming tray until completely dry, or o.n.
28. Dry slides were stored at 4°C until PALM procedure.

PALM Procedure and Extraction of RNA with Problems and Tips for Collecting Samples

29. To collect shoot apical meristems (SAMs) from paraplast embedded sections, shortly before collection of cell tissue the slides were warmed up to RT and sections were de-paraffinized in two changes of xylene (10 minutes each xylene change). One slide was removed from the xylene (the remainder of the slides were left in the xylene until needed), let air-dry, and placed on the microscope stage to mark tissue regions to be collected.
30. Tissue was catapulted into inverted tube-caps (500 µl microcentrifuge tubes), which were filled with about 50 µl RNA extraction buffer (e.g. XB from the PicoPure RNA Isolation kit). After collecting sufficient amount of tissue RNA is extracted according to each RNA extraction protocol.
31. Some of RNA extraction buffers dry out very quickly under dry weather conditions. Crystals form on the outside of the collection tube cap and will eventually touch the slide

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with the tissue. This may cause the rest of the liquid buffer to flow onto the slide and ruin the sample. To avoid this ~40 µl mineral oil may be used as a substitute for RNA extraction buffer (M. Scanlon, personal communication). Tissue is collected into the mineral oil. After collection, RNA extraction buffer is added to the mineral oil and mixed thoroughly. The remainder of the RNA extraction procedure may be performed as if there were no mineral oil.

Materials and Suppliers

Materials	Supplier	Catalog Number
Acetone A.C.S.	Fisher Scientific Pittsburgh, PA	A18-4
Xylene A.C.S.	Fisher Scientific Pittsburgh, PA	X5-4
Diethylpyrocarbonate (DEPC)	Sigma	D5758
Paraplast + 56 °C	Oxford St. Louis, MO	8889-502004
Scintillation vials (20 ml)	Fisher Scientific Pittsburgh, PA	03-340-4E
Tissue Tek metal base molds 22x22x6	Electron Microscopy Sciences Hatfield, PA	62527-22
Tissue Tek embedding rings	Electron Microscopy Sciences Hatfield, PA	62350
Slide warmer	Fisher Scientific Pittsburgh, PA	12-594
“homemade” metal hot plate	Iowa State Chemistry Department of Chemistry Shop	Best replaced with an embedding center
“homemade” rotator	Iowa State Chemistry Department of Chemistry Shop	A similar rotator is offered by “Ted Pella Inc”
P.A.L.M MicroBeam 115V Z	P.A.L.M. Microlaser Technologies Bernried, Germany	
Rotary Microtome	Leica	Type: RM 2135
PicoPure RNA Isolation Kit	Arcturus	kit0202 (10 columns) or Kit0204 (40 columns)