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Plant Genomics

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PREPARATION OF PLANT TISSUE FOR LASER MICRODISSECTION

(Last Revised: August, 2010)

This protocol was developed by the Schnable Laboratory (Iowa State University). Please contact Dr. Patrick Schnable (schnable@iastate.edu) regarding questions or corrections.

Two protocols have been established for preparing plant tissue for laser microdissection.

FROZEN SECTIONING PROTOCOL

This protocol was developed by David Skibbe based on protocols published by Nakazono et al., 2003. The frozen sectioning protocol is divided into two sections: fixation/cryoprotection and cryosectioning. The frozen sectioning protocol described below was optimized for microdissecting the tapetal cell layer from maize anthers. As with many protocols, this protocol is intended to serve as a starting point for other researchers interested in microdissecting cells of interest and may require further optimization for other tissue types. Recipes for solutions and a list of the materials and suppliers used for optimization are in Appendices A and B, respectively.

Nakazono M, Qiu F, Borsuk LA, Schnable PS (2003) Laser-capture microdissection, a tool for the global analysis of gene expression in specific plant cell types: identification of genes expressed differentially in epidermal cells or vascular tissues of maize. Plant Cell, 15:583-596.

SECTION 1 – FIXATION AND CRYOPROTECTION

TISSUE PROCESSING PROCEDURE

1. Place the tissue (ca. 5 mm sections) in a scintillation vial containing approximately 10 ml of 3:1 ethanol:acetic acid fixative and vacuum infiltrate on ice at 400 mm Hg for 20 minutes.
2. Swirl the fixative in the vials to ensure that the tissue is submerged in fixative and incubate at 4°C for 1 hour. The incubation can be performed either with or without rotating.
3. Remove the fixative either by decanting or with an RNase-free Pasteur pipette.
4. Repeat steps 1 and 2 one time and then infiltrate at 4°C overnight.
5. Remove the fixative by either decanting or with an RNase-free Pasteur pipette.
6. Add approximately 10 ml of 10% sucrose and vacuum infiltrate on ice at 400 mm Hg for 15 minutes.
7. Swirl the vials to ensure that the tissue is submerged in cryoprotectant and incubate at

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4°C for 1 hour.

8. Add approximately 10 ml of 15% sucrose and vacuum infiltrate on ice at 400 mm Hg for 15 minutes and incubate for a minimum of 1 hour (or overnight[1]). Remove the 15% sucrose by either decanting or with an RNase-free Pasteur pipette.
9. Fill an appropriately sized Tissue-Tek® cryo-mold (e.g. 15 mm x 15 mm x 5 mm) nearly full with Tissue-Tek® Optimal Cutting Temperature (O.C.T.) compound and orient the sample as desired.
10. Remove all air bubbles by lifting them to the surface of the O.C.T. and moving them to the corners of the cryo-mold with fine-point forceps or a probe.
11. Freeze the embedded sample in liquid nitrogen by placing it in a floating plastic lid (e.g., the lid to a standard pipette tip box).
12. Store the samples at -80°C[2].

SECTION 2 – CRYOSECTIONING

CRYOSECTIONING PROCEDURE

All sectioning steps described in this procedure were performed using a Leica CM1850 Cryostat (Leica Microsystems, Bannockburn, IL). Tissue sections were collected and affixed to slides using the CryoJane system (Instrumedics, Hackensack, NJ).

1. Equilibrate the block to be cryosectioned at -21°C (in the cryostat chamber) for 1 to 2 hours.
2. Prepare 70% ethanol, 95% ethanol, 100% ethanol, and xylene. Pour 50 ml of each reagent into baked, glass staining jars. Place one jar of 70% ethanol, 95% ethanol, 100% ethanol, and two jars of xylene on ice. Keep one jar of 70% ethanol and one jar of xylene at room temperature[3].

NOTES FOR FIXATION AND CRYOPROTECTION

[1] Step 8: Lengthening the cryoprotection steps may improve the morphology of the tissue. Lengthening the 10% sucrose cryoprotection step from 1 hour to 2 days and the 15% sucrose cryoprotection step from overnight to 4 to 7 days significantly improved morphology of the tapetal cell layer of maize. RNA was successfully isolated, amplified, and hybridized to a cDNA microarray where cryoprotection treatments under these conditions.

[2] Step 11: RNA has been isolated, amplified and successfully hybridized to a cDNA microarray after 8 months of storage at –80°C.

NOTES FOR CRYOSECTIONING

[3] Step 2: These reagents should be prepared immediately before use. Dilute the ethanol with diethylpyrocarbonate-threaded, autoclaved water.

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3. Apply O.C.T. compound to the chuck and spread using the heat extractor. Allow the O.C.T. to completely freeze before proceeding.
4. Apply a small drop of O.C.T. compound to the spread O.C.T. base, press the sample block firmly to the base, and spray with CytoCool II rapid freezing aerosol spray. Allow the mounted block to stand for approximately 2 minutes before sectioning.
5. Cool the Instrumedics CryoJane system adhesive-coated slides and tape windows at -21°C in the cryostat prior to sectioning[4].
6. Set the section thickness to 20 µm and trim the block until the tissue becomes visible.
7. Decrease the section thickness to 6 to 12 µm and cut three to four sections to smooth the face of the block.
8. Place an adhesive-coated slide on the temperature-sensitive pad.
9. Peel the cover from a tape window, adhere the window to the block face, and roll it flat.
10. Cut the section and place it on the slide section-side down, and affix the sample to the slide using a cold roller.
11. Place the slide in the UV irradiation chamber and cure the adhesive with one flash of UV light.
12. [5]Peel the tape window off of the slide. The sample should remain affixed to the slide.
13. Place the slide in a staining jar containing 70% ethanol for 5 to 10 minutes at room temperature.
14. Transfer the slide to a staining jar containing 70% ethanol for 2 minutes on ice.
15. Transfer the slide to a staining jar containing 95% ethanol for 2 minute on ice.
16. Transfer the slide to a staining jar containing 100% ethanol for 2 minutes on ice.
17. Transfer the slide to a staining jar containing xylene for 2 minutes on ice.
18. Transfer the slide to a second staining jar containing xylene for 2 minutes on ice.
19. Transfer the slide to a third staining jar containing xylene at room temperature.
20. Take out a slide, air-dry and begin microdissection, or else, if you cannot finish:
21. Store the slides at 4°C[6].

NOTES FOR CRYOSECTIONING

[4] Step 5: Pre-cooling improves the adhesion properties of the CryoJane system.

[5] Step 12: Batch-processing can be performed by repeating steps 12 and 13 until the maximum capacity of the staining jar is reached (i.e., 9 or 15 slides).

[6] Step 20: Slides that were stored at 4°C for up to ten days have yielded RNA with quality sufficient for RNA amplification and CDNA microarray hybridization.

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APPENDIX A – SOLUTIONS

Table 1. List of solutions for the frozen sectioning procedure:

Solution	Purpose
3:1 ethanol:acetic acid	Fixation
10% sucrose	Cryoprotection
15% sucrose	Cryoprotection
1X PBS	Buffer for cryoprotection solutions
70% ethanol	Sample dehydration
95% ethanol	Sample dehydration
100% ethanol	Sample dehydration
Xylene	Sample dehydration and storage

3:1 Ethanol:Acetic Acid

Prepare this solution immediately prior to fixation. Bottles of denatured absolute ethanol can be pre-cooled by storing at 4°C. Prepare 500 ml of the 3:1 ethanol:acetic acid by mixing 375 ml of cold absolute ethanol with 125 ml of concentrated glacial acetic acid.

Cryoprotection solutions

1. Prepare 300 ml of 1X Phosphate Buffered Saline (PBS) for each cryoprotection solution as described in Table 1.

Table 2. Recipe for preparing 1X PBS.

		Molecular Weight (g)	Molarity	Molecular Formula	Amount
1X PBS	Sodium chloride	58.44	0.137	NaCl	2.4 g
	Sodium phosphate (dibasic)	141.96	0.00801	Na_2PO_4	0.34 g
	Potassium chloride	74.55	0.00268	KCl	0.06 g
	Potassium phosphate (monobasic)	136.09	0.00147	KH_2PO_4	0.06 g
	Autoclaved, DEPC-treated water				Up to 300 ml

2a. To prepare the 10% (w/v) sucrose solution, add 30.0 g of sucrose to 300 ml of 1X PBS.
2b. To prepare the 15% (w/v) sucrose solution, add 45.0 g of sucrose to 300 ml of 1X PBS.
NOTE: These cryoprotection solutions can be stored at 4°C for up to one month.

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Table 3. Recipes for preparing the ethanol solutions for cryosectioning.

Solution	# staining jars	Volume absolute ethanol	Volume DEPC-treated dH_2O	Total Volume
70% ethanol	2	70 ml	30 ml	100 ml
95% ethanol	1	47.5 ml	2.5 ml	50 ml
100% ethanol	1	50 ml	0 ml	50 ml

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APPENDIX B – MATERIALS AND SUPPLIERS

Although specific suppliers are included, this does not indicate that materials/equipment from other suppliers would not be suitable; we simply have not tested them.

Table 1. List of materials used in cryosection protocol.

Materials/Equipment	Supplier	Catalog Number
20 ml borosilicate glass scintillation vials, case of 500	Fisher Scientific Hanover Park, IL	03-337-4
Adhesive-coated slides, 125/box	Instrumedics Hackensack, NJ	CJ-1x
Cytocool II rapid freezing aerosol, 11 oz/can, 12/case	Fisher Scientific Hanover Park, IL	22-050-123
Diethylpyrocarbonate, 50 ml	Sigma St. Louis, MO	D5758
Ethyl alcohol USP, Absolute—200 proof, 1 pint plastic bottle	Aaper Alcohol Shelbyville, KY	N/A
Glacial acetic acid, certified ACS Plus, 2.5 L	Fisher Scientific Hanover Park, IL	BP2401-212
Glass staining jars	Electron Microscopy Sciences Hatfield, PA	70316-02
Leica CM1850 Cryostat	Leica Microsystems Bannockburn, IL	Leica CM1850
Potassium chloride, crystalline, certified ACS, 3 kg	Fisher Scientific Hanover Park, IL	P217-3
Potassium Phosphate, monobasic, crystalline, certified ACS, 3 kg	Fisher Scientific Hanover Park, IL	P285-3
Sodium Chloride, crystalline, certified ACS, 3 kg	Fisher Scientific Hanover Park, IL	S271-3
Sodium Phosphate, dibasic, anhydrous, certified ACS, 3 kg	Fisher Scientific Hanover Park, IL	S374-3
Sucrose, crystalline, certified ACS, 3 kg	Fisher Scientific Hanover Park, IL	S5-3
Tape windows, 400/roll	Instrumedics Hackensack, NJ	TW
Tissue-tek cryo-mold, intermediate, 15 mm x 15 mm x 5 mm; 100/pk	Electron Microscopy Sciences (Hatfield, PA)	62534-15
Tissue-Tek® Accu-Edge® disposable, high profile microtome blades	Fisher Scientific Hanover Park, IL	NC9527669
Tissue-Tek® O.C.T. Compound, 4 oz.	Electron Microscopy Sciences Hatfield, PA	62550-01
Xylenes, certified ACS, 4L	Fisher Scientific Hanover Park, IL	X5-4