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Typha (Cattail) Crosslinking and Nuclei Isolation

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ABSTRACT

Typha Crosslinking and Nuclei Isolation protcol

Key notes:

- Allow for extra time if working with pre-ground samples. Crosslinking steps and subsequent washes will take longer to complete.
- 1% Triton X-100
- 600g pelleting
- Optional supernatant recovery steps

PROTOCOL CITATION

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KEYWORDS

Crosslinking, Grass, typha, typha latifolia, typha domingensis, poales, nuclei isolation, cattails, cattail

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36744

MATERIALS

NAME	CATALOG #	VENDOR
Isopropanol		
Potassium Chloride		
HEPES	H6147	Sigma Aldrich
MgCl2		
Weighing Paper, Square, 13/8X13/8X5/16, 200Sheets/Box	WT121.SIZE.1PK	Bio Basic Inc.
Sucrose	S7903	Sigma Aldrich
Razor blade		
Miracloth	475855	Merck Millipore
2-Mercaptoethanol	M3148	Sigma Aldrich
glycine		Sigma
Triton X-100	85112	Fisher Scientific
Glycerol	17904	Thermo Fisher Scientific

NAME	CATALOG #	VENDOR
formaldehyde	F79	Fisher Scientific
PMSF Protease Inhibitor	36978	Thermo Fisher
Cryo Gloves	4426TS	Thermo Fisher
CoorsTek Porcelain Pestle 262mm L	CP1782132	Thermo Fisher
Protease Inhibitor Cocktail (for plant cell and tissue extracts)	9599	Sigma-aldrich
Porcelain Mortar CoorsTek	6882E46	Thomas Scientific

EQUIPMENT

NAME	CATALOG #	VENDOR
Vacuum Desiccator	2246	
Laboratory Tubing	1183A33	Thomas Scientific
No. 8 Perforated Stopper	1211G53	Thomas Scientific
Vacuum Pump	WOB-L Pump 2522	
PYREX Vacuum Filter Flask	4948H29	Thomas Scientific
CHEF Screened Caps	1703711	BioRad Sciences

SAFETY WARNINGS

Resuspend anhydrous Magnesiem Chloride carefully. Anhydrous MgCl2 is very hygroscopic (absorbs water readily) and can flash boil.

Always use cold resistant gloves when working with liquid nitrogen.

DISCLAIMER:

BEFORE STARTING

Determine if you will have time to complete both the Crosslinking and Nuclei Isolation steps. Always make the Nuclei Isolation Buffer on the same day that you will perform the prep. If you are working with pre-ground material, leave extra time to complete the crosslinking steps.

Remember to have liquid nitrogen handy for flash freezing tissue after crosslinking.

Buffers

1 **Formaldehyde Buffer**

1% Formaldehyde
Water

Final Volume 50mL

2 **125mM Glycine**

125mM Glycine
Water

Final Volume 50mL

3 **Nuclei Isolation Buffer (NIB)**

Prepare this buffer the same day that you will perform the nuclei isolation.

Resuspend anhydrous Magnesiem Chloride very slowly and carefully. Anhydrous MgCl2 is very hygroscopic (absorbs water readily) and can flash boil.

Prepare 30mL of NIB, chilled to 4C, per sample:

250mM Sucrose
20mM HEPES pH 8.0
5mM Potassium Chloride
1mM Magnesium Chloride
40% Glycerol

Just before use, add the following:
0.1mM PMSF
1% Protease Inhibitor Cocktail
0.1% 2-mercaptoethanol
1% Triton X-100

Bring the final volume to 30mL with water.

				For 1 RXN:	
Reagent	**Stock Concentration**	**Final Concentration**		**Stock Amount**	**Unit**
Sucrose		250mM		2.56	g
HEPES pH 8.0	1M	20mM		600	uL
Potassium Chloride (KCl)	1M	5mM		150	uL
Magnesium Chloride (MgCl2)	1M	1mM		30	uL
Glycerol	100%	40%		12	mL
Water				14.23	mL
100mM PMSF Solution	100mM	0.1mM		30	uL
Protease Inhibitor Cocktail (Sigma #P8340)	100%	1%		300	uL
2-mercaptoethanol	100%	0.10%		750	ul
Triton X-100	10%	1.00%		3	mL
			Total Volume	30	mL

Equipment

4

Laboratory Tubing
Tygon® S3™ E-3603 Laboratory Tubing

Tygon 1183A33

No. 8 Perforated Stopper
Stopper

MilliporeSigma 1211G53

Vacuum Pump

Welch WOB-L Pump 2522

Any vacuum that can apply the correct pressure will work for this protocol

PYREX Vacuum Filter Flask
Flask

Pyrex 4948H29

CHEF Screened Caps
Cap

BioRad 1703711

Vacuum Desiccator

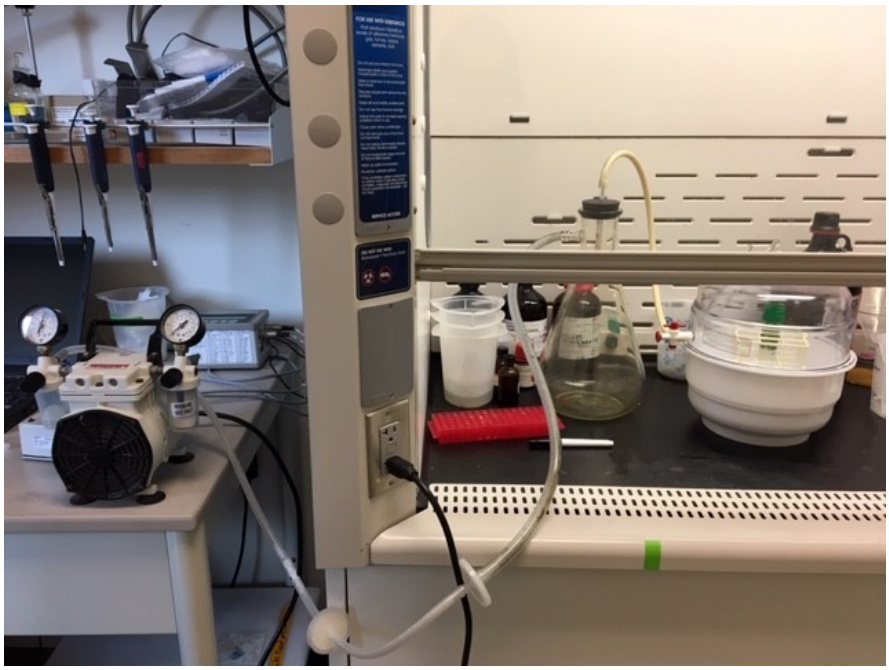
Ted Pella 2246

Any vacuum desiccator that will fit your samples will work

Crosslinking

5 In a chemical hood, prepare a vacuum dessication setup. Ensure that the valve on the dessication chamber is open and attach the hose coming from the flask to the nozzle. On the outside of the hood, attach the hose coming from the

erlenmeyer catch flast to the vaccum pump.

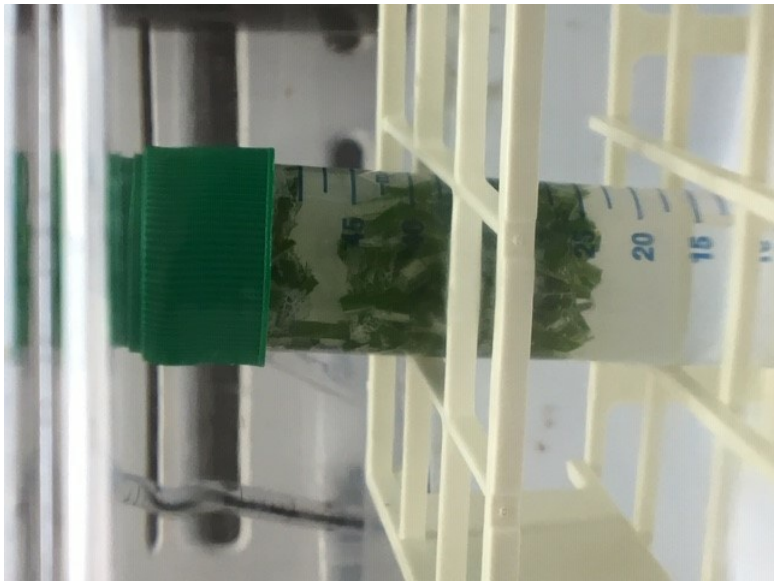


6 Weigh out 2-6g of tissue. Using a sterile razor blade cut the tissue into small, approximately 1 cm X 1 cm pieces.

If you are working with pre-ground tissue, use more if possible. You will lose more tissue in the initial crosslinking steps.

7 Once all buffers and equipment are prepared, put the sample in a cotton bag, and place in a 50mL conical. The cotton bag helps to keep all of the tissue uniformly submerged in the buffers. Though not idea, if you do not have a cotton bag, use weigh paper to keep all tissue submerged in the buffers.

For pre-ground tissue*; Do not put pre-ground tissue in a cloth bag.*



In this example, weigh paper was used to attempt to block cut tissue from floating to the top of the buffer during vacuum incubation.

8 Add 50 mL crosslinking buffer, ensuring the tissue is fully submerged. Use the screened cap to allow air pressure to change in the tube, but keep the original 50mL tube cap, you will use it in the wash steps.

9 Put the tube in the tube rack, and place in the dessicator + replace the lid. Vacuum incubate for 10 min at 30cm Hg (400milibar) pressure. A small amount of bubbleing should be visible. **Stay and watch the pressure on the pump the entire time, or until pressure stabilizes.**

10 Release vacuum pressure by turning the pump off. Remove the dessicator lid and pour out the crosslinking buffer though the screened cap into the appropriate hazardouse liquid waste container.

- ***For pre-ground tissue*** *- use miracloth or 100uM cell strainter to filter the crosslinking buffer off of the sample. It will likely become clogged so allow for extra time. Change gloves between samples to prevent cross contamination after touching the miracloth.*

11 Add 50mL 125mM glycine, make sure the tissue is fully submerged, and replace the screened cap. Make sure the original cap is not on the tube, air pressure needs to change in this step. Put the tube in the tube rack, and place in the dessicator + replace the lid. Vacuum incubate for 5 min at 30cm Hg (400milibar) pressure. A small amount of bubbling should be visible.

12 Release vacuum pressure by turning the pump off. Remove the dessicator lid and pour out the 125mM glycine in the appropriate hazardous liquid waste container.

- ***For pre-ground tissue*** *- Again, use miracloth or100uM cell strainter to filter the glycine buffer off of the sample. Change gloves between samples to prevent cross contamination after touching the miracloth.*

13 Add 50mL milliq/di water, make sure the tissue is fully submerged, replace the original cap on top of the screened cap, and mix. Repeat this process three times to fully wash the tissue. Change gloves if processing multiple samples to prevent cross contamination.

- ***For pre-ground tissue*** *- Again, use miracloth or 100uM cell strainter to filter the water off of the sample. Complete all washes for one sample at a time to reduce the number of glove changes.*

14 Transfer the plant tissue to aluminum foil and fold it up. Try to allow any remaining water to run off of the sample.

Snap-freeze the sample by submerging the aluminum foil containing the tissue in liquid nitrogen.

Safe Stop

15 Store at -80C until ready to proceed to nuclei isolation.

Nuclei Isolation

16 **Before Starting, aquire:**

Liquid Nitrogen
Dry Ice
Cold Resistant Gloves
Ceramic Mortar and Pestle
Miracloth, cut into ~7cm x 7cm pieces
Cool a large centrifuge to 4C

Ensure the sucrose in the nuclei isolation buffer is fully resuspended, and the solution has been chilled on ice.

17 Place a mortar and a 50mL conical tube onto a bed of dry ice, and place a pestle into the mortar. Pour liquid nitrogen into the mortar until the entire pestle tip is submerged. Allow liquid nitrogen to evaporate completely.

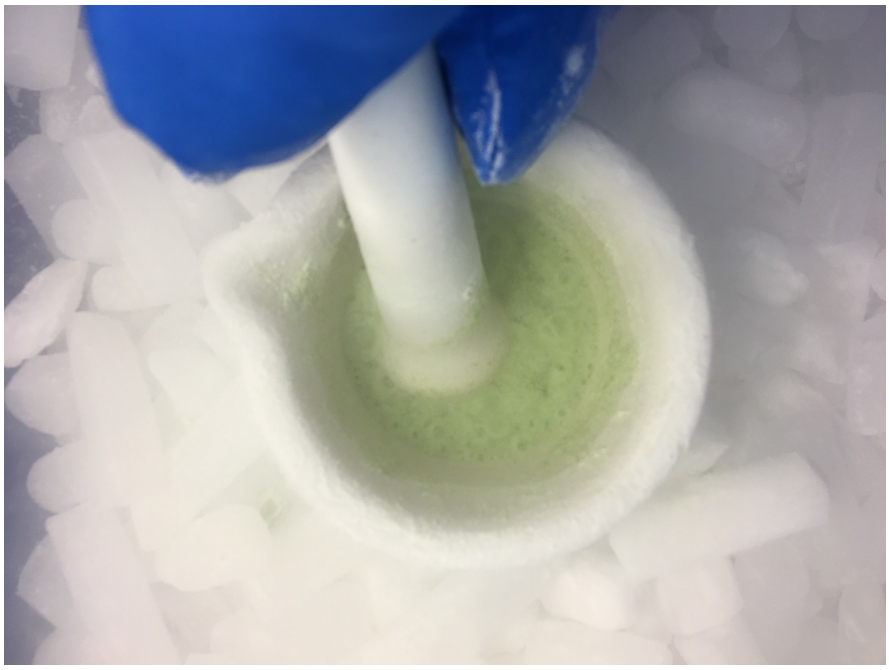
Cool a spatula at -20°C or colder for later use.

18 Pour liquid nitrogen into the mortar until the entire pestle tip is submerged. Transfer frozen plant tissue into mortar with liquid nitrogen.

19

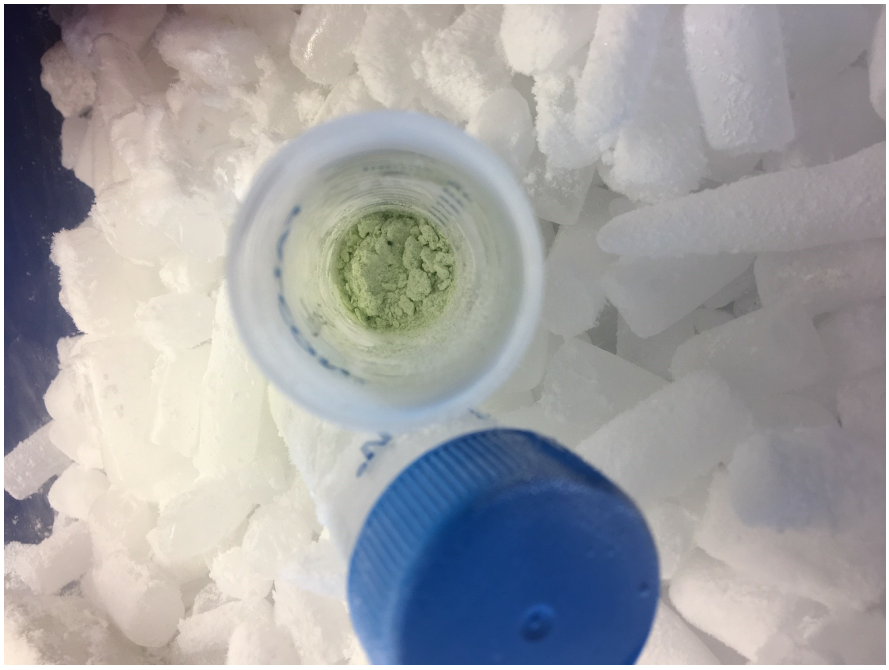
Put on protective cryo gloves.

Pulverize plant tissue in the mortar with the pestle until the sample resembles a fine powder. Ensure the plant tissue is always submerged in liquid nitrogen. Carefully re-fill the mortar with liquid nitrogen as necessary. The pulverization process should take at least 5 min per sample. The key is to pulverize until the tissue resembles a fine powder without visible chunks.



20 Once the sample resembles a fine powder, allow liquid nitrogen in the mortar to evaporate just enough for sample to stay submerged. Carefully pour pulverized plant tissue and remaining liquid nitrogen from the mortar into the pre-chilled 50mL conical tube. Ensure the tube does not overflow with liquid nitrogen.

21 Using the pre-cooled spatula, transfer any remaining pulverized plant tissue from the mortar into the 50mL tube. Place 50mL conical tube on dry ice to keep the pulverized plant tissue frozen. **DO NOT CAP** the 50mL conical until all of the liquid nitrogen has evaporated.



Note the powder-like appearance, and the tissue has lightened in color

Safe Stop

22 Crosslinked, ground tissue may be stored at -80C until ready to proceed to continue nuclei isolation

Nuceli Isolation

23 Add the PMSF, Protease Inhibitor Cocktail, Triton X-100, and 2-mercaptoethanol to the chilled nuclei isolation buffer, and bring the volume to 30mL with water.

24 Add 20mL Nuclei Isolation Buffer to the pulverized plant tissue and mix gently by inversion

25 Place a 10cm X 10cm section of Miracloth over a new 50mL conical tube and insert the miracloth down into the tube, creating a small basket.

26 Pour the pulverized plant tissue over the miracloth. The Nuclei will pass through the cloth while the larger plant debris will be caught.

Optional: *Pipette 1mL of NIB over the tissue caught by the miracloth to wash more nuclei through.*

27 Place another 10cm X 10cm section of Miracloth over a new 50mL conical tube, and pour the filtrate over the miracloth. Repeat this step once more for a total of three miracloth filtrations.

28 Discard the Miracloth and place a cap on the 50mL tube containing the nuclei. Wipe down the tube(s) and then pellet by centrifugation at 600 g at 4C for 15 min.

29 ***OPTIONAL:***

Save the supernatant for each step! Nuclei can be lost in the process of pouring supernatant off or if that sample needs a different centrifugal force. It's possible to recover a small amount of nuclei if the main pellet yeild looks very low.

Pour off supernatant, into new 50mL conical or into the appropriate hazardous waste container.

30 Resuspend pellet in 2mL NIB Buffer. Pellet sample by centrifugation at 600 g at 4C for 10 min.

31 Pour off supernatant into designated supernatant reservoir or into the appropriate hazardous waste container.

32 Resuspend pellet in 1mL NIB.

33 ***OPTIONAL:***

- *If the nuclei pellet is still green, re-filter the sample through miracloth and pellet at 600 g at 4C for 10 min*
- *If you are concerned with yeild and want to recover any possible lost nuclei from the combined supernatants, centrifuge supernatant at 600 g at 4C for 10 min. Pour off and discard the supernatant. Resuspend the pellet (if there is one) in 500 uL NIB and move into a 1.5mL tube. Centrifuge the tube at 600g for 10 minutes. Pour off and discard the supernatant. Resuspend the pellet (if there is one) in 500 uL NIB, and centrifuge the tube at 600g for 10 minutes. Pour off and discard the supernatant. Now resuspend the pellet in up to 1mL NIB and combine with the*

main nuclei pellet.

34 Make aliquots of the resuspended nuclei:

- Make a small aliquot of the pellet for the to determine the yeild - 100 to 200uL should work. Be concious of the size of your pellet, you don't want to use too much for QC if possible. Record the volume of this aliquot and stock volume, you will need it to accurately back calculate to determine total yeild.
- If you have a very large pellet, you may also make multiple "stock" aliquots. Record the number and volume of each aliquot so you can determine the amount of nuclei in each

35 Pellet the nuclei at 600 g at 4C for 10 min.

36 Discard supernatant and resuspend each aliquot in 500uL NIB for the final wash step.

37 Pellet the nuclei at 600 g at 4C for 10 min. Discard the supernatant leaving behind only a nuclei pellet and minimal residual liquid. Freeze samples on dry ice or liquid nitrogen and store at -80C.