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Optical mapping preps for Petunia spp.

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1 Works for me dx.doi.org/10.17504/protocols.io.baq3idyn

High molecular weight DNA extraction from all kingdoms Plantae view 1 more group

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ABSTRACT

Optical mapping technologies assist the assembly of large and complex genomes. Although protocols for preparing this kind of samples are now conveniently available as kits, some plants will not respond to standardized conditions or the researcher might not have access to kits.

This protocol describes how to isolate plant nuclei from fresh very young leaves. The nuclei are purified over two Percoll® gradients and embedded in agarose. The nuclear DNA is released by solubizing the nuclear membrane with detergents and removing the proteins by digestion with Proteinse K. The nuclear DNA is analyzed by pulse field gel electrophoresis. If most of the DNA is > 1 Mbp, the sample could be further evaluated for optical mapping analyzis by the service provider.

GUIDELINES

Consumables and equipment

- Live young very fresh leaves
- Scissors
- Scalpel blades
- Glass Petri Dishes, sterile
- Tweezers
- Squirt bottle with 99% ethanol from a bulk supply for cleaning purposes
- Low lint tissues
- 15 mL Corning™ Falcon tubes with rack
- 50 mL Corning™ Falcon tubes with rack
- Green screened cap for 50 mL Falcon tubes
- Glass tissue grinder, tapered, 15 mL
- Disposable plastic Pasteur pipettes
- Glass Pasteur pipettes with dropper bulb
- Miracloth sheets cut 10 x 10 cm
- Small funnel, washed and rinsed with ethanol
- Spatulas with flat and round end points, washed and rinsed with ethanol
- Cell strainers, 100 and 40 µm
- 1.5 mL screw capped tubes, sterile
- 1.5 mL microcentrifuge tubes, sterile
- Benchtop centrifuge with swing bucket rotor, precooled at 10°C with adaptors for 15 and 50 mL conical tubes
- Benchtop microcentrifuge for 1.5 mL tubes
- Ice bucket with crushed ice
- Water bath at **70 °C**
- Hot block set at **43 °C** (check temperature with a thermometer inmersed in one well filled with water)
- Hybridization oven set at **50 °C**
- Sterile deionized water

MATERIALS

NAME	CATALOG #	VENDOR
Sodium Hydroxide	BP359500	Fisher Scientific

NAME	CATALOG #	VENDOR
Tween 20	170-6606-MSDS	Bio-rad Laboratories
PIPES KOH	View	Sigma Aldrich
D-Mannitol	View	Sigma Aldrich
Polyvinylpyrrolidone K40	View	Sigma Aldrich
L-Lysine monohydrochloride	View	Sigma Aldrich
EGTA	View	Sigma Aldrich
Magnesium chloride hexahydrate	View	Sigma Aldrich
Sodium metabisulfite	View	Sigma Aldrich
2-Mercaptoethanol	View	Sigma Aldrich
Triton X-100	T8787-50ML	Sigma Aldrich
Percoll	17-0891-01	Sigma Aldrich
CleanCut Agarose	1703594	Bio-rad Laboratories
50-Well Disposable Plugs	1703713	Bio-rad Laboratories
N-lauryl sarcosine	L5125-50G	Sigma Aldrich
Tris(hydroxymethyl)aminomethane	252859-500G	Sigma Aldrich
Certified Megabase Agarose	1613108	Bio-rad Laboratories
Glycine	50046	Sigma
Proteinase K	19131	Qiagen
Miracloth	475855	Merck Millipore
Formaldehyde solution	F8775-25ML	Sigma Aldrich
Green Screened Caps	1703711	BioRad Sciences
15 mL Tissue Grinder Tapered Gnd	358115	
Cell Strainer 40 µm	View	Sigma Aldrich
Cell strainer 100 µm	View	Sigma Aldrich
SYBR™ Safe	S33102	Thermofisher
Ethylenediaminetetraacetic acid	E9884	Sigma Aldrich

SAFETY WARNINGS

- Work in a fume cabinet
- Consult your institution's policy on chemical waste collection and management

BEFORE STARTING

1. Nuclei isolation

- **Nuclei extraction buffer (NEB)**

	g/L
Mannitol	91

PIPES-KOH	3.78
$MgCl_2.7H_20$	2
L-lysine monohydrochloride	36.5
EGTA	2.28
PVP-K40	20

Nuclei extraction buffer (NEB): 0.5 M Mannitol, 10 mM PIPES-KOH, 10 mM $MgCl_2$, 2% PVP-K40, 200 mM L-lysine monohydrochloride, 6 mM EGTA, pH 6

Adjust to pH 6 with 10 M NaOH, split in two 1 L bottles (500 mL each), autoclave, and store at 4°C

- Prepare these buffers and keep them on ice:

	NEB Complete	NEB -βME
Nuclei extraction buffer (NEB)	50 mL	50 mL
sodium metabisulfite	0.1 g	0.1 g
β-mercaptoethanol	20 µL	--

- **1X PBS buffer:** 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, 500 mL sterile and cold
- **Fixing solution**: 56.8 mL 1X PBS + 3.2 mL Formaldehyde solution (Sigma-Aldrich F8775-25ML), keep cold
- **0.125 M Glycine pH ~ 5** Dissolve 0.937 g of glycine in 100 mL sterile deionized water, do not adjust the pH, and keep cold
- **25% Triton X-100** : Mix 750 µL NEB complete plus 250 µL mL Triton X-100. Vortex and spin down until all is dissolved

2. Percoll gradient buffers

- **NEB -PVP/M/T:** 0.5 M Mannitol, 10 mM PIPES-KOH, 10 mM $MgCl_2$, 200 mM L-lysine monohydrochloride, 6 mM EGTA, pH 7.0, sterile. Prepare 1 L
- **Percoll**® Once opened, store at 4°C
- Prepare these buffers and keep them on ice:

	NEB_A	NEB_B
NEB -PVP/M/T	60 mL	50 mL
sodium metabisulfite	0.12 g	0.1 g
β-mercaptoethanol	120 µL	--
Triton X-100	300 µL	--

3. Agarose plugs

- CleanCut 2% Agarose
- 50 µL-Well Disposable Plug Molds

4. Proteinase K digestion solutions

- **2X Proteinase K lysis buffer (2X TLS)**, sterile: 20 mM Tris-HCl pH 8, 400 mM L-lysine monohydrochloride, 2% lauryl sarcosine, pH 8.0
- 0.5 M EDTA pH 8
- Proteinase K (20 mg/mL, Qiagen)

- **Proteinase K lysis mix** Prepare fresh before starting

2X TLS	3 mL
0.5 M EDTA pH 8	3 mL
Proteinase K 20 mg/mL	396 µL

Plug Wash buffers

	Wash-1	Wash-2
2X TLS	25 mL	25 mL
0.5 M EDTA pH 8	2.5 mL	0.5 mL
Sterile deionized water	22.5 mL	24.5 mL
Na-metabisulfite	0.1 g	--

Tissue Fixation

1

Do the tissue fixation and nuclei isolation steps in a fume hood

Remove ~ **0.5 g very young leaves** and place them in a 50 mL Falcon tube containing ~ **45 ml Fixing solution** .
Attach the green screened cap and slowly add more Fixing solution (total volume 60 mL).
Remove all the air bubbles by tapping the tube but keeping it upright. Close the tube with its own cap and incubate on ice.
0 °C Formaldehyde fixing reaction **00:10:00 Formaldehyde fixing reaction**

2 Discard the Fixing solution by pouring it through the green screened cap.

Dispose the formaldehyde waste according to your institution's waste management guidelines.

3 Add **60 ml 0.125 M glycine solution** through the green screened cap, and remove the air bubbles as in step 1. Incubate on ice. **0 °C Fixing quenching step** **00:10:00 Fixing quenching step**

4 Discard the glycine solution through the green screened cap. Add **60 ml ice cold 1X PBS solution** and remove air bubbles as in step 1. Incubate on ice **0 °C 1X PBS wash** **00:10:00 1X PBS wash** . Repeat this step two more times.

Nuclei isolation

5 After decanting the last 1X PBS wash, transfer the leaf tissue to the Petri dish containing ~ **4 ml ice cold NEB complete** .
Place the Petri dish securely on ice. Finely chop the sample using two scalpel blades, as if they were knife and fork until the pieces are ~ 1 mm.

6 Transfer the sample including the buffer to the tissue homogenizer using a spatula and a disposable Pasteur pipette. Pour 5-10 mL of ice cold NEB complete into the homogenizer, insert the pestle and place it on the ice bucket so it is covered by ice up to half way of its length.
Homogenize the sample by genlty grinding with the pestle in a spiral movement, and lift it just below the liquid level. Avoid making foam. If too much tissue stays at the bottom of the homogenizer carefully take out the pestle and use a disposable Pasteur pipette to bring the sample up into the medium. Expect a fairly homogeneous mixture, with no foam.

Alternatively, the sample can be ground with liquid nitrogen.

After the last 1X PBS wash, place the tissue on a clean Petri dish and pat dry with a lint-free tissue. Transfer the sample to a precooled mortar and freeze with liquid nitrogen. Do 3 cycles of grind/freeze until you obtain a fine powder (like icing sugar). Transfer the powdered sample to a 100 mL glass beaker containing a magnetic stirrer and 30 mL NEB-complete buffer. Stir gently until an homogeneous mixture is obtained. Proceed with step 7.

If you have access to other grinding equipment, like a bead beater or a TissueLyzer with a grinding jar, follow the instrument's instructions.

7 Place one sheet of Miracloth inside the funnel and place it in a 50 mL Falcon tube. Pre-wet the Miracloth with 2-3 mL NEB complete. Discard the filtered solution.

8 Filter the homogenized sample through the Miracloth sheet. If the flow is too slow, gently brush the inside of the sheet with a clean spatula. Discard the Miracloth sheet and adjust the volume to **30 ml ice cold NEB complete** .

9 Add **600 µl 25% Triton X-100** by pipetting it at the top of tube's inside wall, in a circular way. Close the tube and mix genlty by invertion, about 20 times. The final concentration of Triton X-100 is 0.5%.

10 Collect the nuclei by centrifugation. **1800 x g , Nuclei pellet** **00:15:00 Nuclei pellet** **10 °C Nuclei pellet**

11 Discard supernatant and gently resuspend the pellet with **30 ml ice cold NEB −βME** by gently inverting the closed tube.

Dispose the supernatant containing β-mercaptoethanol according to your institution's guidelines.

12 Spin down as before **go to step #10 Nuclei pellet** . Discard the supernatant and resuspend the nuclei pellet in **7 ml NEB_A** by gently swirling the tube and keep on ice.

Percoll® gradients

13 While waiting for the two centrifugations described above, prepare the Percoll® gradients:

- Prepare **10 ml 75% Percoll® in NEB_A** and split into two 15 mL Falcon tubes. The two tubes should weigh exactly the same.
- Prepare 10 mL of **10 ml 37.5% Percoll® in NEB_A** 37.5% , split into two 15 mL Falcon tubes. The two tubes should weigh exactly the same.
- Keep both sets in the refrigerator until ready.

The buffers used for preparing the Percoll® gradients do not contain PVP-K40. In plants rich in polyphenols PVP can form a brown precipitate in the final agarose plug that prevents the DNA to migrate freely out of the agarose matrix.

14 Assemble two 50 mL Falcon tubes with the 100 and 40 µm sieves, respectively, and place securely in on the ice bucket.

15 Pour the nuclei suspension **go to step #12 Nuclei suspension in NEB-A** through the 100 µm sieve, and then through the 40 µm sieve. Discard the sieves. Keep sample on ice.

If the flow through the 40 µm sieve slows down, replace it with a new sieve.

16 Collect nuclei as before **go to step #10 Nuclei pellet** . Discard supernatant and resuspend the nuclei pellet in **7 ml NEB_A** 7 mL NEB_A. Repeat this step **three** more times.

17 Carefully overlay **3.5 ml filtrated nuclei suspension** over each 75% Percoll solution. The loaded tubes should weigh **exactly** the same.

18 Collect the nuclei at the Percoll® gradient interphase by centrifugation **650 x g , Percoll gradient** **00:30:00 Percoll gradient** **10 °C Percoll gradient** . Adjust the acceleration = 9 (max), and deceleration = 1 (min).

19 With a light source behind the gradient, collect the sample found at the interphase of the Percoll® gradient using a glass Pasteur pipette. Do this step very slowly. Transfer sample to a new 15 mL Falcon tube and keep on ice.

20 Carefully overlay half of the sample on each 37.5% Percoll® tube. Each tube should weight **exactly** the same. Collect the nuclei by centrifugation **go to step #18 Percoll gradient** .

21 Remove the solution with a disposable Pasteur pipette without disturbing the nuclei pellet, which is small and has a pale yellowish color.

22 Resuspend each nuclei pellet in 10 mL NEB_B and collect by centrifugation. **650 x g , Nuclei wash after gradients** **00:30:00 Nuclei wash after gradients** **10 °C Nuclei wash after gradients** . Both acceleration and deceleration set at 9 (max).

23 Remove the solution with a disposable Pasteur pipette without disturbing the nuclei pellet but leave ~ **200 µl** of solution. Gently tap the bottom of the tube to resuspend the nuclei and transfer to one 1.5 mL microcentrifuge tube. Repeat the same with the other nuclei pellet. Add ~ **600 µl NEB_B** and spin down at 6,000 rpm, 1 min at room temperature.

24 Discard **600 µl supernatant** and resuspend the nuclei in the remaining **400 µl NEB_B** by tapping the bottom of the tube. Keep on ice until ready.

Agarose plugs

25 While running the Percoll® gradients, place the CleanCut™ 2% Agarose bottle in the water bath at **70 °C** . When the agarose is completely melted, transfer **400 µl** to a screw-capped tube, and leave it in the water bath until ready.
Precool one strip of disposable plug molds by sitting on a piece of Parafilm placed over the ice bucket.

26 Incubate the nuclei at **43 °C** , and the melted 2% agarose for **00:10:00 nuclei + agarose mix** .

27 Transfer the nuclei mix to the agarose tube, mix very gently by pipetting in and out until completely even.

28 Place precooled plug mold on the bench, over a paper towel.

29 Dispense ~ **90 µl** per plug. Prepare ~ 8 plugs. Cool down completely **0 °C Plugs setting** **00:10:00 Plugs setting** .

30 Pop out the agarose plug with the plastic tab at the end of the plug mold into the Proteinase K lysis mix at **50 °C Proteinase K digestion** **20:00:00 Proteinase K digestion** without shaking.

31 Wash plugs in **25 ml Wash-1** at **50 °C** for **01:00:00** without shaking.

32 Wash plugs in **25 ml Wash-2** at **20 °C** for **01:00:00** in a rotisserie at slowest speed. Place tube horizontally. The tube will rotate on its side, like a cabin of a Ferris wheel.

33 Store each plug in ~ **2 ml Wash-2** in screw capped tubes and store at **4 °C** . Avoid introducing air bubbles in the tube.

34 Analyze plugs by PFGE: 1% agarose gel (Certified™ Megabase Agarose BioRad) in 1X TAE. Settings: 60-120", 120°, 6 V/cm, **15 °C** , **24:00:00** . Stain gel with SYBR™ Safe.