Optical mapping preps for kiwifruit

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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 in kits, some plants won't respond to the standardized conditions or the researcher might not have access to it.

Here I provide a draft protocol I have been working on for kiwifruit, with limited success. The nuclear genomic DNA produced is ~ 1 Mbp long but it has not passed the service provider QC step.
I would be very happy to hear from the ResearchGate and Protocos.io communities regarding any interest in collaborating to improve this method and eventually make it available to a wider audience.

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Guidelines

Consumables and equipment

- Sprouted kiwifruit canes grown in the glasshouse, with young leaves and plenty of roots
- Scissors
- Fine hair paint brush
- Scalpel blades
- Plastic Petri Dishes
- Tweezers
- 15 mL Falcon tubes with rack
- 50 mL Falcon tubes with rack
- Tissue homogenizer, ~ 7 mL capacity
- Disposable plastic Pasteur pipettes
- Glass Pasteur pipettes with bulb
- Miracloth sheets cut 10 x 10 cm
- Rubber bands
- Spatulas with flat and round end points
- SS34 rotor with sterile OakRidge tubes, and centrifuge, precooled at 10°C
- Falcon cell strainers, 100 and 40 µm (Fisher Scientific cat# 08-771-19 and 08-771-1)
- Benchtop centrifuge with swing bucket rotor, precooled at 10°C
- Benchtop microcentrifuge with 1.5 mL Eppendorf and screw-cap tubes
- Water bath at 70°C and hot block calibrated at 43°C

- Hybridization oven set at 50°C

Before start

Prepare the following buffers:

1. Nuclei isolation

- Nuclei isolation buffer (Otto I)

0.1 M citric acid, 0.5% Tween 20 (v/v), pH 2-3, prepared in sterile deionized water, cooled on ice.

- Nuclei extraction buffer (NEB) Complete

0.5 M Mannitol, 10 mM PIPES-KOH pH 6, 10 mM $MgCl_2$, 2% PVP-K40, 200 mM L-lysine monohydrochloride, 6 mM EGTA, 10 mM sodium metabisulfite and 5 mM β-mercaptoethanol (in percentage v/v is equal to 0.04%).

	g/L
Mannitol	91
PIPES-KOH	3.78
$MgCl_2.7H_20$	2
L-lysine monohydrochloride	36.5
EGTA	2.28
PVP-K40	20

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

Before using this buffer, add 0.1 g of sodium metabisulfite to two 50 mL aliquots, in Falcon tubes. Add 20 µL β-mercaptoethanol to only one tube, now labelled as NEB Complete.

- 25% Triton X-100

750 µL NEB complete plus 250 µL mL Triton X-100. Vortex and spin down until all is dissolved.

- NEB - βME

The other 50 mL NEB tube containing sodium metabisulfite, but no β-mercaptoethanol.

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

- Percoll GE Healthcare, cat# 17-0891-01. Once opened, store at 4°C.
- NEB-A

NEB -PVP/M/T	50 mL
Na-metabisulfite	0.1 g
β-mercaptoethanol	100 µL
Triton X-100	250 µL

- NEB-B

NEB-PVP/M/T	50 mL
Na-metabisulfite	0.1 g

3. Agarose plugs

- CleanCut 2% Agarose, BioRad cat# 1703594
- 50-Well Disposable Plug Molds, BioRad #1703713

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

- Proteinase K (20 mg/mL, Qiagen cat# 19133)
- Proteinase K lysis mix

2X TLS	3 mL
0.5 M EDTA pH 8	3 mL

Prepare fresh by adding 396 µL Proteinase K

- Wash-1

2X TLS	25 mL

0.5 M EDTA pH 8	2.5 mL
ddH_2O	22.5 mL
Na-metabisulfite	0.1 g

- Wash-2

2X TLS	25 mL
0.5 M EDTA pH 8	0.5 mL
ddH_2O	24.5 mL

Materials

Citric Acid View by P212121

Proteinase K, Molecular Biology Grade - 2 ml P8107S by New England Biolabs

Sodium Hydroxide BP359500 by Fisher Scientific

Tween 20 170-6606-MSDS by Bio-rad Laboratories

PIPES KOH View by Sigma Aldrich

D-Mannitol View by Sigma Aldrich

Polyvinylpyrrolidone K40 View by Sigma Aldrich

L-Lysine monohydrochloride View by Sigma Aldrich

EGTA View by Sigma Aldrich

Magnesium chloride hexahydrate View by Sigma Aldrich

Sodium metabisulfite View by Sigma Aldrich

2-Mercaptoethanol View by Sigma Aldrich

Triton X-100 T8787-50ML by Sigma Aldrich

Percoll 17-0891-01 by Sigma Aldrich

CleanCut Agarose 1703594 by Bio-rad Laboratories

50-Well Disposable Plugs 1703713 by Bio-rad Laboratories

N-lauryl sarcosine L5125-50G by Sigma Aldrich

Tris(hydroxymethyl)aminomethane 252859-500G by Sigma Aldrich

Ethylenediaminetetraacetic acid E9884-500G by Contributed by users

Certified Megabase Agarose 1613108 by Bio-rad Laboratories

Protocol

Nuclei isolation

Step 1.

Remove 1 g of tissue (young leaves or root tips thoroughly rinsed in tap water) and place them in a 15 mL Falcon tube containing 10 mL Otto I buffer. If using roots, place the root tips on 5 mL Otto I buffer poured into a Petri dish, sitting on ice. Use a fine hair paint brush to remove all debris. When ready, transfer the root tips to the Falcon tube containing 10 mL of Otto I buffer.

Step 2.

Incubate the sample on ice for 10 min, horizontally, with gentle shaking. If too many bubbles accumulate between the leaves, tap the tube over the bench to release them. Check again half way through the incubation for bubbles.

Step 3.

Pour out the buffer and transfer the sample into a Petri dish with 5 mL of Otto I buffer. Work inside the fume hood.

Step 4.

Finely chop the sample using two scalpel blades, as if they were knife and fork until the pieces are 1 mm.

Step 5.

Transfer the sample to the tissue homogenizer, including the buffer, using a spatula and a disposable Pasteur pipette. Pour 5-10 mL of NEB Complete into the homogenizer and insert the pestle. Homogenize the sample by pulling and pushing the pestle, gently and just below the liquid level, in a spiral movement. Avoid making foam. If too much tissue stays at the bottom of the homogenizer, take out the pestle and use a disposable Pasteur pipette to bring the sample up into the medium.

Step 6.

Place one sheet of Miracloth over the mouth of a 50 mL Falcon tube, depress the centre with your thumb, and attach it to the tube with rubber bands. Prewet the Miracloth with 2-3 mL NEB Complete.

Step 7.

Filter the homogenized sample through the Miracloth sheet. If the flow slows down, gently brush the

inside of the sheet with a clean spatula. Discard the Miracloth sheet and adjust the volume to 30 mL with NEB Complete.

Step 8.

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

Step 9.

Transfer the sample to an OakRidge tube, and spin down at 3,380 rpm (1,800 g, SS34 rotor) for 15 min at 10°C.

Step 10.

Discard supernatant and gently resuspend the pellet with 30 mL NEB –βME by gently inverting the closed tube.

Step 11.

Spin down as before. Discard the supernatant.

Percoll gradients

Step 12.

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

Prepare 10 mL of 75% Percoll in NEB-A and split into two 15 mL Falcon tubes. The two tubes should weight exactly the same. Prepare 10 mL of 37.5% Percoll in NEB-A, split into 2 tubes. The two tubes should weight exactly the same. Keep both sets in the fridge until ready.

NOTES

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The buffers used for preparing the Percoll gradients do not conain PVP-K40 because it forms a brown precipiate in the final agarose plug that prevents the DNA to migrate freely out of the matrix. However the isolated nuclei obtained in step 10 are dissolved in NEB-βME, at about 1% PVP-K40, which dilutes into the gradient buffers.

Step 13.

Resuspend the pellet in 7 mL NEB-A by gently swirling the tube.

Step 14.

Assemble two 50 mL Falcon tubes with the 100 and 40 µm sieves, respectively.

Step 15.

Pour the sample throuugh the 100 µm sieve, and then through the 40 µm sieve. Keep the sample on ice.

Step 16.

Carefully overlay 3.5 mL of nuclei solution over each 75% Percoll solution. The loaded tubes should weight exactly the same.

Step 17.

Spin down at 650 g, 30 min at 10°C. Acceleration = 9 (max), deceleration = 1 (min).

Step 18.

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 very slowly. Transfer sample to a new 15 mL Falcon tube. Repeat the same with the second 75% Percoll gradient tube. The total sample should be 4 mL. Gently resuspend the sample until homogeneous.

Step 19.

Carefully overlay half of the sample on each 37.5% Percoll tube. Each tube should weight exactly the same. Spin down as before.

Step 20.

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

Step 21.

Resuspend each nuclei pellet in 10 mL NEB-B and spin down at 650 g, 30 min at 10°C. Both acceleration and deceleration set at 9 (max).

Step 22.

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 a 1.5 mL Eppendorf tube. Repeat the same with the other nuclei pellet. Add 600 µL of NEB-B and spin down at 6,000 rpm, 1 min at room temperature.

Step 23.

Discard 600 µL of 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

Step 24.

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 on ice, sitting on a piece of Parafilm.

Step 25.

Incubate the nuclei at 43°C, and the melted 2% agarose for 10 min.

Step 26.

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

Step 27.

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

Step 28.

Dispense 90 µL per plug. Prepare 8 plugs. Cool down completely for 10 min on ice.

Step 29.

Pop out the agarose plug with the plastic tab at the end of plug mold into the Proteinase K lysis mix for 20 h at 50°C without shaking.

Step 30.

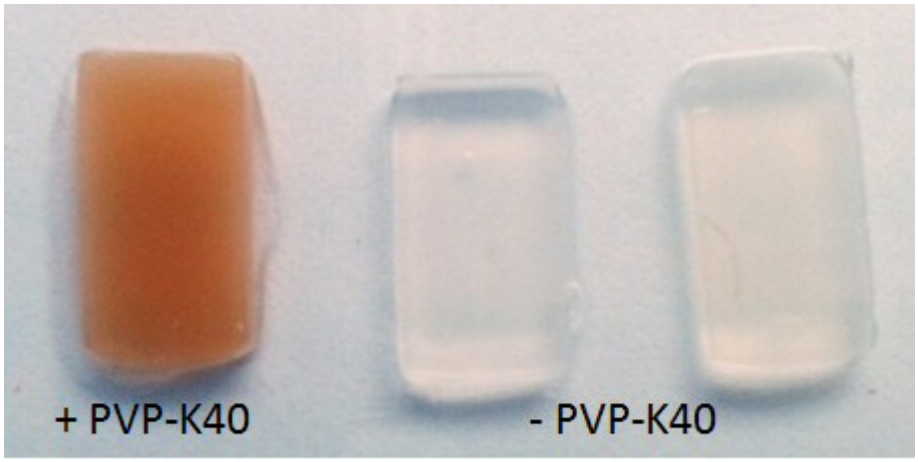
Wash plugs in 25 mL Wash-1 for 1 h at 50°C without shaking.

Step 31.

Wash plugs in 25 mL Wash-2 for 1 h at room temperature in a rotisserie at slowest speed.

Step 32.

Store plugs in 2 mL Wash-2, at 4°C.



Step 33.

Analyze plugs by PFGE: 1% agarose gel (Certified™ Megabase Agarose BioRad cat #1613109) in 1X TAE. Settings: 60-120”, 120°, 6 V/cm, 15°C, 24 h. Stain gel with SybrSafe.