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Dianthus (Caryophyllaceae) high-molecular weight genomic DNA extraction protocol for PacBio sequencing

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We use this protocol and it's working

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Abstract

This protocol is an adaptation of the method optimized by Miriam Schalamun and Benjamin Schwessinger, based on **Mayjonade et al. 2016**, available at **Protocols.io**. The objective of this protocol is to obtain exceptionally clean, high–molecular weight DNA from leaf samples of the *Dianthus* genus for PacBio sequencing. The protocol has been optimized and tested on *D. caryophyllus* L. and *D. broteri* Boiss. & Reut.

Materials

- PVP 40
- PVP 10
- NaCl
- Tris pH8
- EDTA
- SDS 20% (10% also works)
- Sodium metabisulfite
- DTT
- RNase A (100mg/mL)
- Proteinase K
- Potassium Acetate
- SPRi Beads (SPRIselect – Beckman Coulter. Other beads can be used but should be
- washed)
- TE buffer
- Sodium Acetate
- Cloroform
- Isoamyl alcohol
- Ethanol

Safety warnings

Do not vortex the samples and avoid spinning them for a long time.

Perform steps involving toxic reagents such as DTT or chloroform in a fume hood and a well ventilated room.

Before start

This protocol is a two-day protocol. The first day of the protocol comprises steps 1 to 31 and the second day comprises steps 32 to 40. If you want to test the protocol on your samples, you can do it in one day only, but results improve significantly with overnight incubation.

Make sure you have all the reagents ready and read the entire protocol before starting the extraction.

If the DNA is pure enough after the extraction steps, cleanup can be skipped (steps 26-40).

IMPORTANT: Do not vortex the samples and avoid spinning them for a long time.

Lysis buffer preparation

30m

1 Always prepare fresh lysis buffer before extraction for optimal results (especially DTT should be added fresh). 30m

For 10 mL Lysis Buffer:

	Final	Stock	Input (10mL)	Input (5mL)
	1% PVP 40 (0.1g in 1ml for stock)	10%	1mL	500µL
	1% PVP 10 (0.1g in 1ml)	10%	1mL	500µL
	500mM NaCl (0.2922g in 1mL)	5M	1mL	500µL
	100mM Tris pH 8	1M	1mL	500µL
	50mM EDTA	0.5M	1mL	500µL
	1.25% SDS (2g SDS in 100mL)	20%	625µL	312.5µL
	1% (w/v)! Sodium metabisulfite	190.1 g/mol	0.1g	0.05g
	5mM Dithiothreitol (DTT) (0.077g in 500µl)	1M	50 µL	12.5µL
	Miliq Water		4.3mL	2.16mL

Heat lysis buffer to 64 °C for 30 minutes. (This is important for the DNase heat inactivation). After cooling down to room temperature, add per 1 mL lysis buffer 1 µL RNase A (in this case 10µL or 5µL).

00:30:00 Heating to 64ºC

10 µL RNAse A

Tissue preparation

2 In the meantime, prepare 2 mL Eppendorf tubes with 2-3 glass beads (4 mm) and 100 mg of tissue and transfer tubes into liquid nitrogen.

This is the easiest with fresh tissue because freeze thawing is avoided during cutting. However, if there is no other way, tissue frozen in liquid nitrogen (ensuring it stays frozen) also works.

100 mg Tissue

(COOL DOWN CENTRIFUGE TO 4ºC AND HEAT UP THE THERMOBLOCK TO 37ºC FOR NEXT STEPS)

Grinding

30s

3 Before grinding make sure tissue is completely frozen in liquid nitrogen and perform the grinding steps as quickly as possible to avoid freeze thawing. The grinding rack can also be frozen to ensure that (it is highly recommended to store it at -80°C for at least 3 hours, overnight if possible). 30s

Grind the tissue using an automated grinder (Qiagen TissueLyzer II) for 30 seconds (actual grinding time may differ from tissue to tissue). Immediately put the tube into liquid nitrogen. If the tissue is not well ground, repeat this step until it is (grinding is crucial for the extraction).

00:00:30 Grinding

Alternatively, grind the frozen material manually with a mortar.

Extraction I

2h 7m

4 Add 700 µL of preheated buffer and mix by inverting tube until no frozen clumps of tissue are left (This can take up to a few minutes, but it's worth it).

700 µL Preheated buffer

5 Incubate at 37°C in a thermomixer shaking at 400 rpm (slowly) for 20 minutes. 20m

00:20:00 Incubation at 37ºC

6 Add 10 µL proteinase K per tube and incubate for another 30 min at 37°C in the thermomixer. 30m

10 µL Proteinase K

00:30:00 Incubation at 37ºC

7 Take the tubes out of the thermomixer and cool down on ice for 5 minutes. 5m

00:05:00 Cool down on ice

8 Add 210 µL (~0.3 volumes) of 5M potassium acetate and mix by inverting the tube 20 times. Then immediately keep on ice at ~4°C for 10 minutes. 10m

210 µL 5M Potassium Acetate

00:10:00 on ice (4ºC)

9 Centrifuge at 6000g for 20 minutes at 4°C. 20m

6000 x g, 4°C, 00:20:00

10 Transfer the supernatant (~600 µL) to a new 1.5 mL tube without disturbing the pellet.

11 Add 1 volume (~600 µL) of the bead solution (make sure beads are at room temperature and well homogenized by vortexing for approximately 30 seconds). 30s

600 µL Beads solution

00:00:30 Vortex

(VORTEX THE BEADS ALONE, DO NOT VORTEX THE SAMPLES CONTAINING THE DNA)

12 Mix by inversion and then incubate on a rotor for 10 minutes at RT. 10m

00:10:00 Incubation on rotor

(In the meantime **PREHEAT TE-BUFFER AT 50°C**)

13 Spin down the tube for 1 second.

14 Place the tube in a magnetic rack for 5 minutes (until beads are stuck to the wall of the tube and solution becomes clear). 5m

00:05:00 Magnetic rack

15 Remove the supernatant without disturbing the beads.

16 Add 1 mL of fresh 70 % ethanol and wait for 30 seconds. 30s

1 mL 70% Ethanol

00:00:30 Wait

17 Remove supernatant without disturbing the beads.

18 Repeat the washing steps 15 - 16 once again. 5m 30s

19 Spin down the tube for 1 second and place the tube on the magnetic rack to remove the remaining ethanol.

20 Let the beads air-dry for 30 seconds, but not longer because this would decrease elution efficiency. 30s

00:00:30 Air drying

21 Add 50 µL (can be changed to the amount needed, between 20 and 100 µL is recommended) of TE buffer preheated to 50°C and resuspend the beads by flicking the tube (ensure they are no longer aggregated).

50 µL TE buffer

22 Incubate the resuspended beads for 10 minutes at room temperature. 10m

00:10:00 Incubation at RT

23 Spin down the tube for 1 second and place the tube in the magnetic rack and incubate for 5 -10 minutes (until solution becomes clear). 10m

00:10:00 Magnetic rack

24 Transfer the supernatant (eluted DNA) into new tube.

Quantification I (Before Washing)

25 Measure DNA concentration with Qubit and absorbance with NanoDrop. A260/280 values should be between 1.8 and 2.0. A260/230 values should be between 2.0 and 2.0.

If the clean up is going to be performed, Qubit measurement is not really necessary at this point.

Clean up

1h 12m

26 Transfer the DNA solutions into one tube and add TE buffer to make up a total volume of 500 µL.

If the extraction is done with multiple tubes at a time (which is usually the case), then after the elution step all the eluted DNA can just be pipetted into one tube.

27 Add 500 µL (1 V) chloroform:isoamylalcohol (24:1) and invert tube about 100 times (2 minutes at least). 2m

500 µL Chloroform:Isoamylalcohol (24:1)

00:02:00 Invertion mix

28 Centrifuge the tubes to separate the phases at 8000g at 4°C for 10 minutes. If the supernatant is still cloudy centrifuge it again at 6000g at 4°C for 10 minutes. 20m

8000 x g, 4°C, 00:10:00

6000 x g, 4°C, 00:10:00 , if still cloudy

29 Transfer the upper phase (DNA) into a new tube and discard the lower chloroform phase.

(Depending on how well the transfer worked out, one round is usually enough but if some of the intermediate phase has been transferred steps 28 -29 can be repeated. In this case centrifuge only once in step 28).

30 Add 50 µL (0.1 V) 3 M sodium acetate (NaAc) and mix by inverting tube 10-20 times.

50 µL 3 M Sodium Acetate

31 Add 1 mL (2 V) of ice cold (-20°C) 100% ethanol and mix by inverting tube carefully a few times. Then, incubate at -20°C overnight.

(Alternatively, samples can be incubated for 1 or 2 hours and perform the DNA extraction in just one day. Longer precipitation gets more DNA but worse purity).

1 mL 100% Ethanol

Overnight Incubatuin at -20°C

32 Centrifuge at 5000g and 4°C for 5 minutes. 5m

5000 x g, 4°C, 00:05:00

(In the meantime **PREHEAT UP TE BUFFER UP TO 50°C**)

33 Pipette supernatant off and make sure to do it on the opposite side from the pellet (usually transparent) is supposed to be. 10m

Save the supernatant and centrifuge it at 5000g and 4°C for 10 minutes. Then continue the following steps of the extraction using them as extra samples (supernatants can be stored at 4°C or -20°C and be used after extraction just in case no pellet was obtained from step 32).

5000 x g, 4°C, 00:10:00

34 Add 1 mL ice cold 70 % ethanol to pellet to wash off salt.

1 µL 70% Ethanol at 4°C

35 Centrifuge at 4500g and 4°C for 15 minutes. 15m

4500 x g, 4°C, 00:15:00

36 Remove supernatant.

37 Repeat steps 35 - 37 once again and make sure all ethanol is pipetted off. 15m

38 Let air dry (for the last removal of ethanol) for 2 - 5 minutes, or until no residue of ethanol remains in the tubes. 5m

00:05:00 Air drying

(Here, drying time is not as critical as in the beads drying step as the DNA pellet should easier dissolve than the beads)

39 Add 50 µL of preheated (to 50°C) TE buffer and elute DNA as long as necessary for the whole pellet to dissolve.

If it's a large pellet, let it dissolve overnight at room temperature.

50 µL TE buffer

Quantification II (After washing)

40 Measure Qubit and Nanodrop values again (as in step 25).

An agarose gel is also highly recommended to ensure the samples are clean and that there is not DNA fragmentation.