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High molecular weight DNA extraction from fungal tissue for the long read PacBio sequencing

Forked from Multi-step high purity high molecular weight DNA extraction protocol from challenging fungal tissues

Reynaldi Darma[1], Megan McDonald[1]

[1]Australian National University

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Solomon Lab - Australian National University

Reynaldi Darma

ABSTRACT

Obtaining high molecular weight DNA from certain materials for PacBio sequencing can be quite challenging because the quality requirement for the input DNA sample is really high. In my case, I need to provide the HMW DNA with these criteria for the library preparation and sequencing:
-Minimum quantity of 20 µg of DNA for PacBio 10-20kb (Blue Pippin kit)
-Pure DNA (has an OD260/OD280 ratio of 1.8 to 2.0 and an OD260/OD230 ratio of 1.8 - 2.2)
-The Qubit to nanodrop concentration ratio should be above 0.5
-In the pippin pulse gel, the DNA sample should be a band above 40kb with minimum DNA degradation

Therefore, a specific DNA extraction should be performed to obtain the high quality of HMW DNA. I performed DNA extraction from fungal pathogen, *Rhynchosporium commune*, with using Nagar and Schwessinger (2018) protocol with a slight modification especially with the amount of materials and reagents. Although I was unable to obtain the perfect HMW DNA in the end, the Sequel PacBio sequencing from that DNA sample yielded 13.47 Gb from 2 flow cells with the N50 of 20.7 kb. The *de novo* assembly from these reads generated 20 gapless contigs with 57.7 Mb genome size.

GUIDELINES

I used Nagar and Schwessinger (2018) protocol with a slight modification in the amount of materials and reagents. My protocol focuses on obtaining 20 µg of pure HMW DNA from fungal tissue for PacBio sequencing.

Reference:
Nagar, R. and Schwessinger, B. (2018). Multi-step high purity high molecular weight DNA extraction protocol from challenging fungal tissues. *Protocols.io*

MATERIALS TEXT

Stock solutions:
Tris 1M pH 8
0.5M EDTA pH 8
NaCl 4M
Polyvinylpyrrolidone (10000 MW) 10%
Polyvinylpyrrolidone (40000 MW) 10 %
Sodium Acetate (NaAc) 3M pH 5.2

CTAB lysis buffer pH 8:
Tris 100mM pH 8
EDTA 20mM
CTAB 2% w/v
NaCl 1.2M

CTAB precipitation buffer pH 8:

Tris 10mM pH 8
EDTA 20mM
CTAB 2% w/v

Other solutions:
Isopropanol 100%
Ethanol 70%
Phenol:Chloroform:Isoamyl Alcohol (25:24:1) Invitrogen

Enzymes:
RNAse A (10mg/ml)
Proteinase K (20mg/ml)

Preparation

1 Make extraction buffer in 50 ml falcon tube (x3):
13 ml of CTAB lysis buffer
1.5 ml 10% PVP 10
1.5 ml 10% PVP 40

2 Scrap fungal tissues from agar plate with scalpel directly without adding water and weight the total fungal cells. I used 1.3 gram wet weight of fungal cells from 9 plates.

3 Prechill mortar, pestle, spatula, and sand (1gr sand / 100 mg fungal tissues) with liquid nitrogen.

Grinding the spores

4 Grind fungal tissues 3-4 times for 10-15 sec bursts adding liquid nitrogen each time. Do not exert excessive pressure while grinding the spores as this may shear the DNA.

5 Transfer the mixture of ground tissues and sand into 3 of 60 ºC pre-heated 50 ml falcon tubes containing extraction buffer as equal as possible.

DNA extraction

6 Add 200 µl proteinase Proteinase K (20 mg/mL) and incubate at RT for 1.5 hours on the orbital shaker with with slow agitation.
01:30:00

7 Add one volume of Phenol:Chloroform:Isoamyl Alcohol (25:24:1) (16 ml) into the extraction tube, mix several times slowly, and centrifuge 5000g for 10 mins at RT.
00:10:00

8 Take the aqueous phase carefully and put it into new 50 mL falcon tube. Add 20µL RNAse A (10mg/ml) to the tube and incubate 30 mins at RT on the orbital shaker with with slow agitation.
00:30:00

9 Add one volume of Phenol:Chloroform:Isoamyl Alcohol (25:24:1) again into the tube, mix several times slowly, and centrifuge 5000g for 10 mins at RT.

00:10:00

DNA precipitation

10 Take the aqueous phase carefully and put it into new 50 mL falcon tube. Add two volume of CTAB precipitation buffer and incubate the tube into the oven rotator with temperature 55 °C for 10-20 minutes (The mixture will be really cloudy).

00:20:00

11 Centrifuge at 7500g for 30 mins at RT and then discard the supernatant.

00:30:00

12 add 5 mL fresh 70% EtOH to the tube, incubate for 15 mins at RT.

00:15:00

13 Centrifuge at 7500 for 20 mins at RT. Discard most of the supernatant and leave around 1 mL of the supernatant on the tube.

00:20:00

14 Resuspend the pellet and gently dislocate the mixture into 1.5 mL tube. Centrifuge at 7500g for 5 mins at RT.

00:05:00

15 Add additional 1 mL of fresh 70% EtOH, mix slowly and centrifuge at 7500g for 5 mins at RT.

00:05:00

16 Discard the supernatant and air dry the pellet for around 15 mins. Add 400 µL of 10 mM tris (pH8) and let the pellet dissolve for overnight at RT or at 4°C.

Overnight

2nd DNA purification (day 2)

17 Check whether the pellet has dissolved. Incubate the tube at 37ºC for 10 -20 mins if the pellet hasn't dissolved yet. Measure the concentration and the purity of the crude DNA with Qubit and Nanodrop.

00:20:00

18 Add 20µL RNAse A into 400 µL crude DNA and incubate for 1 hour at RT on the orbital shaker with with slow agitation.

01:00:00

19 Add 40µL Proteinase K to each tube and incubate for 1 hour at RT on the orbital shaker with with slow agitation.

01:00:00

20 Add PCI (460 µL) to each tube, mix slowly several times. Centrifuge 8000g for 5 mins.
00:05:00

21 Combine the aqueous phase from all tubes together into one tube (they might have milky upper phase) and adjust the total volume of the aqueous phase into 5 mL with tris 10mM pH 8.

22 Add 1 volume of PCI (5 mL) and mix slowly. Centrifuge 5000g for 10 mins. Collect the aqueous phase from the centre of the tube slowly. (It's possible to have a milky layer in the inner surface of the tube). I usually take 3 mL from the aqueous phase. Additional PCI treatment can be performed to the aqueous phase if the milky layer can't be separated from that aqueous phase.
00:10:00

23 Then add 0.1 volume of 3M NaAc pH 5.2 (300 µL) and mix slowly.

24 Add 1 volume of isopropanol (3.3 mL) and mix slowly.

25 Centrifuge 7500 g for 20 mins. Discard the supernatant and add 5 ml of fresh 70% EtOH. incubate the mixture for 15 mins.
00:35:00

26 Centrifuge at 7500 for 20 mins at RT. Discard most of the supernatant and leave around 1 mL of the supernatant on the tube.
00:20:00

27 Resuspend the pellet and gently dislocate the mixture into 1.5 mL tube. Centrifuge at 7500g for 5 mins at RT.
00:05:00

28 Add additional 1 mL of fresh 70% EtOH, mix slowly and centrifuge at 7500g for 5 mins at RT.
00:05:00

29 Discard the supernatant and air dry the pellet for around 15 mins.
00:15:00

30 Add 500 µL of 10 mM tris (pH8) and let the pellet dissolve for overnight at RT or at 4°C.
Overnight

AMPure beads clean up and size selection (day 3)

31 Check whether the pellet has dissolved. Incubate the tube at 37ºC for 10 -20 mins if the pellet hasn't dissolved yet. Measure the concentration and the purity of the crude DNA with Qubit and Nanodrop.
00:20:00

32 Perform 0.45x AMPure XP beads clean up and size selection.

33 Check the concentration and the purity of the DNA with Qubit and Nanodrop after the AMPure XP beads treatment.

34 Run pulsed-field gel electrophoresis to see the size of the DNA.

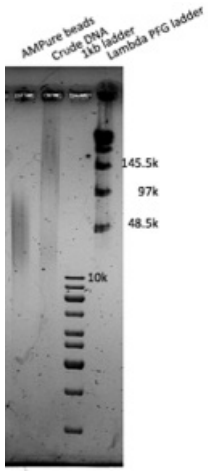
QC analysis

35 This is the concentration and purity of DNA from different steps.

Sample (step)	Qubit (ng/ul)	Nanodrop (ng/ul)	260/280	260/230	Qubit/ nanodrop ratio	Volume (ul)	Total DNA (ug)
Crude DNA	87.6	316	1.83	1.55	0.28	1200	105.1
2nd purification	42.2	68	1.94	1.83	0.62	500	21.1
AMPure beads	305	341.15	1.84	1.74	0.89	60	18.3

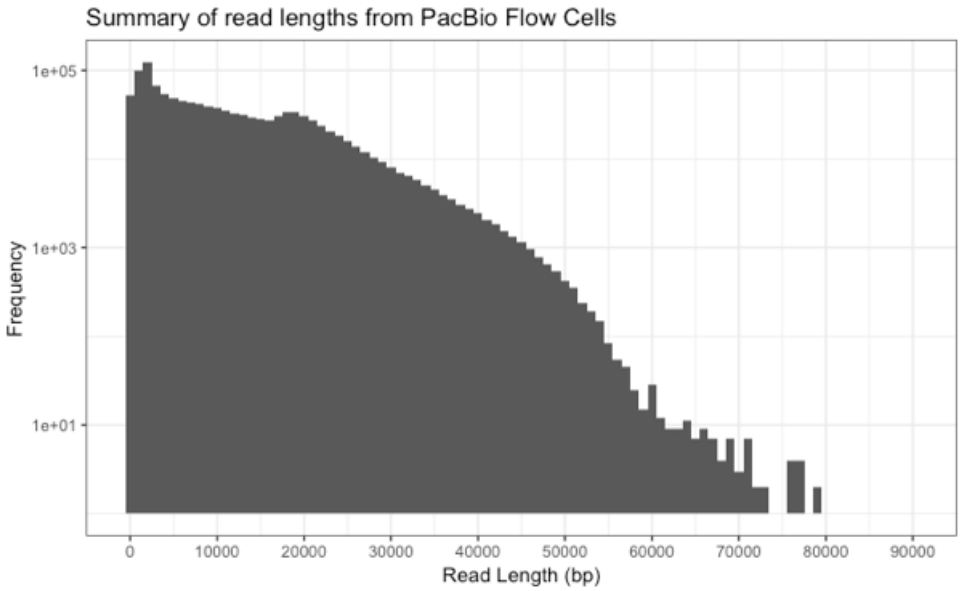
The final AMPure beads DNA has a concentration below 20µg and the 260/230 ratio below 1.8. However, I still used it for the sequencing.

36 The pulsed-field gel showed the size of the majority of DNA after AMPure XP beads treatment was around 10kb - 97kb.



PacBio sequencing

37 The Sequel PacBio sequencing from the clean DNA sample yielded 13.47 Gb from 2 flow cells with the N50 of 20.7 kb.



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