High quality DNA from Fungi for long read sequencing e.g. PacBio Version 10

Benjamin Schwessinger

Abstract

Extraction of high quality DNA for long read sequencing e.g. PacBio

Optimized for DNA extraction from wheat stripe rust spores and also tested on barley leaf rust.

Buffers are best when fresh and not older than 3-6 months. Buffered Phenol:Chloroform:Isoamylalcohol (25:24:1) should not be older than 3 months.

Critical steps to obtain high quality DNA:

- Do NOT heat samples during DNA extractions! Perform all steps at RT or 4oC as indicated.
- Do NOT incubate samples with KAc for prolonged time periods
- Perform two steps of buffered Phenol:Chloroform:Isoamylalcohol purification to reduce co-purifying metabolites.

DNA fragments were well above the 40kb mark based on Pippin Pulse Gels. The sequencing center performed a second AMPure purification step before library construction. Summary statistics of sequencing runs to follow.

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Guidelines

Modified from protocols of Prof. Pietro Spanu (Imperial College, London) and T. M.

Fulton, J. Chunwongse, S. D. Tanksley, Pl Mol Biol Rep 13, 207 (1995)

I am gratefull for critical suggestion from the following scientists.
Dr. Claire Anderson, Dr. Andril Gryganskyi, and Dr. David Hayward.

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Reagents required

BUFFER A: 0.35 M sorbitol

0.1 M Tris-HCl, pH 9

5 mM EDTA, pH 8

autoclave to sterilize

BUFFER B: 0.2 M Tris-HCl, pH 9

50 mM EDTA, pH 8

2 M NaCl

2% CTAB

autoclave to sterilize

BUFFER C: 5% Sarkosyl (N-lauroylsarcosine sodium salt SIGMA L5125)

Filter-sterilize

Other solutions:

Potassium Acetate 5M (KAc precipitate polysaccharides) pH 7.5

Polyvinylpyrrolidone (40000 MW) 1 % [w/v] (Sigma PVP40)

Sodium Acetate (NaAc) 3M pH 5.2

Filter-sterilize

Isopropanol 100%

Ethanol 70%

Buffered Phenol:Chloroforme:Isoamylalcool P:C:I (25:24:1, Sigma P2069)

Autoclave acid washed Sand

Enzymes

RNAse T1 (1000 U/ml, Thermo Fisher EN0541)

Proteinase K (800U/ml, NEB P81072)

AMPure beads from Beckman

Lysis Buffer For 17.5 ml for 500 mg starting material

2.5 volume of Buffer A 6.5 ml

2.5 volume of Buffer B 6.5 ml

1.0 volume of Buffer C 2.75 ml

PVP 0.1 % 1.75 ml

Protocol

Extraction I

Step 1.
Make lysis buffer by mixing buffer A+B+C (2.5:2.5:1 + 0.1%PVP final) and briefly heat to 64 °C. Let cool to room temperature for use in 50mL Falcon tubes.
All following steps are based on 17.5ml lysis buffer as starting volume.

Extraction I

Step 2.
add 10uL (10kU) RNAse T1 to lysis buffer

Extraction I

Step 3.
Grind tissue/spores with liquid nitrogen in a mortar with sand, use 1g of sand per 100mg of starting material. Grind for 2 mins in 4x 15 sec bursts adding liquid nitrogen after each 15 sec grinding burst.

Extraction I

Step 4.

Transfer powder to 50mL Falcon containing lysis buffer and RNAse, mix well by vortexing

Extraction I

Step 5.

Incubate at RT for 30 mins mixing by inversion every 5 mins

DURATION

00:30:00

Extraction I

Step 6.

Add 200uL Proteinase K, incubate at RT for 30 mins mixing by inversion every 5 mins

DURATION

00:30:00

Extraction I

Step 7.

Cool on ice for 5 mins

DURATION

00:05:00

Extraction I

Step 8.

Add 3.5 mL (0.2 vol) of KAc 5M, mix by inversion, incubate on ice for max 5 mins

Extraction I

Step 9.

Spin at 4°C and 5000g for 12 mins

DURATION

00:12:00

Extraction I

Step 10.

Transfer supernatant to fresh Falcon tube containing 17.5ml (1vol) (P/C/I) and mix by inversion for 2 mins

DURATION

00:02:00

Extraction I

Step 11.

Spin at 4 °C and 4000g for 10 mins

DURATION

00:10:00

Extraction I

Step 12.

Transfer supernatant (might be milky but do not worry) to fresh Falcon tube containing 17.5ml (1vol) P/C/I and mix by inversion for 2 mins

DURATION

00:02:00

Extraction I

Step 13.

Spin at 4 °C and 4000g for 10 mins

DURATION

00:10:00

Extraction I

Step 14.

Transfer supernatant (17mL) to fresh Falcon tube and add 5uL RNAse T1

Extraction I

Step 15.

Incubate for 20-30mins at RT

DURATION

00:20:00

Extraction I

Step 16.

Add 1.8mL (0.1vol) NaAc and mix by inversion

Extraction I

Step 17.

Add 18mL (1vol) RT isopropanol and mix by inversion

Extraction I

Step 18.

Incubate at RT for 5-10mins

DURATION

00:05:00

Extraction I

Step 19.

Spin at 4 °C and 10000g for 30 mins

DURATION

00:30:00

Extraction I

Step 20.

Carefully pipette off supernatant till about 1-2 mL left, DNA will form a mostly translucent to white film/pellet at the bottom of the tube

Extraction I

Step 21.

Use 1mL pipette tip to transfer pellet and remaining liquid into fresh 1.7mL eppendorf tube. If the pellet got loose during transfer add 1.5mL fresh 70% EtOH to the 50mL Faclon and spin for 5min at 4000g. Remove 1mL and transfer the remaining volume and DNA pellet to same 1.7mL eppendorf tube.

Extraction I

Step 22.

Spin in table top centrifuge for 5 mins at 13000g

DURATION

00:05:00

Extraction I

Step 23.

Remove supernatant with pipette and wash with 1.5mL fresh 70% Ethanol, invert several times to dislodge pellet

Extraction I

Step 24.

Spin in table top centrifuge for 5 mins at 13000g

DURATION
00:05:00

Extraction I

Step 25.
Remove supernatant with pipette and wash with 1.5mL fresh 70% Ethanol, invert several times to dislodge pellet

Extraction I

Step 26.
Spin in table top centrifuge for 5 mins at 13000g

DURATION
00:05:00

Extraction I

Step 27.
Remove supernatant with pipette

Extraction I

Step 28.
Spin in table top centrifuge for 1 min at 13000g

DURATION
00:01:00

Extraction I

Step 29.
Remove remaining ethanol with pipette

Extraction I

Step 30.
Air-dry pellet for 7 mins

DURATION
00:07:00

Extraction I

Step 31.
Add 200uL of 10mM Tris pH9 leave at RT for 3 hours

DURATION
03:00:00

Extraction I

Step 32.
Flick tube slightly for mixing and add 200uL of TE buffer. DO NOT! vortex as it shears DNA.

Extraction I

Step 33.
leave at RT over night

DURATION
16:00:00

Extraction I

Step 34.
Next day add another 100uL TE buffer and incubate for 1h at 28 °C with 1400rpm shaking

DURATION
01:00:00

Extraction I

Step 35.
Measure dsDNA concentration using BR Qubit and measure absorbance with Nanodrop. At this point Qubit to Nanodrop ratios were 1/10 -1/20.
This might be also a good step to assess DNA quality by runing a 0.8% TBE agarose gel with 500ng dsDNA and a lamda-Hind-III ladder as control. If you have a Pulse Field Gel Electrophoresis around even better.

NOTES

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next step is 40. This has come out of order and will be fixed.

Extraction II

Step 36.
Use AMPure beads for secondary clean up at beads 0.45 (Vol/Vol) following the PacBio protocol.

Extraction II

Step 37.

Elute in 10mM Tris pH8

Extraction II

Step 38.

Measure dsDNA concentration using BR Qubit and measure absorbance with Nanodrop

Extraction II

Step 39.

At this stage Qubit to Nanodrop ratios were 0.64, 260/280 1.87 and 260/230 1.37

Results

Step 40.
Samples were submitted to Ramaciotti (http://www.ramaciotti.unsw.edu.au/) sequencing centre in Sydney. Excellent personel performed quality control, prepared 15-20kb libraries and we ran 13 SMRT cells with P6 chemistry. Some summary statistics are shown below.

EXPECTED RESULTS

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Summary statistics for all 12 SMRT cells combined

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 Total sequence amount:                          1.263691e+10
 Number of reads:                                1.181543e+06
 Median read length:                                     9632
 Mean read length:                                      10695
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Pippin Pulse Gel: 9hr 10-48kb 0.75% KBB gel