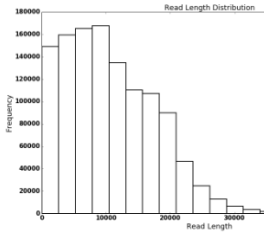
May 17, 2019 Version 11

High quality DNA from Fungi for long read sequencing e.g. PacBio V.11



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Protocol status: Working
We use this protocol and it's working

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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.

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

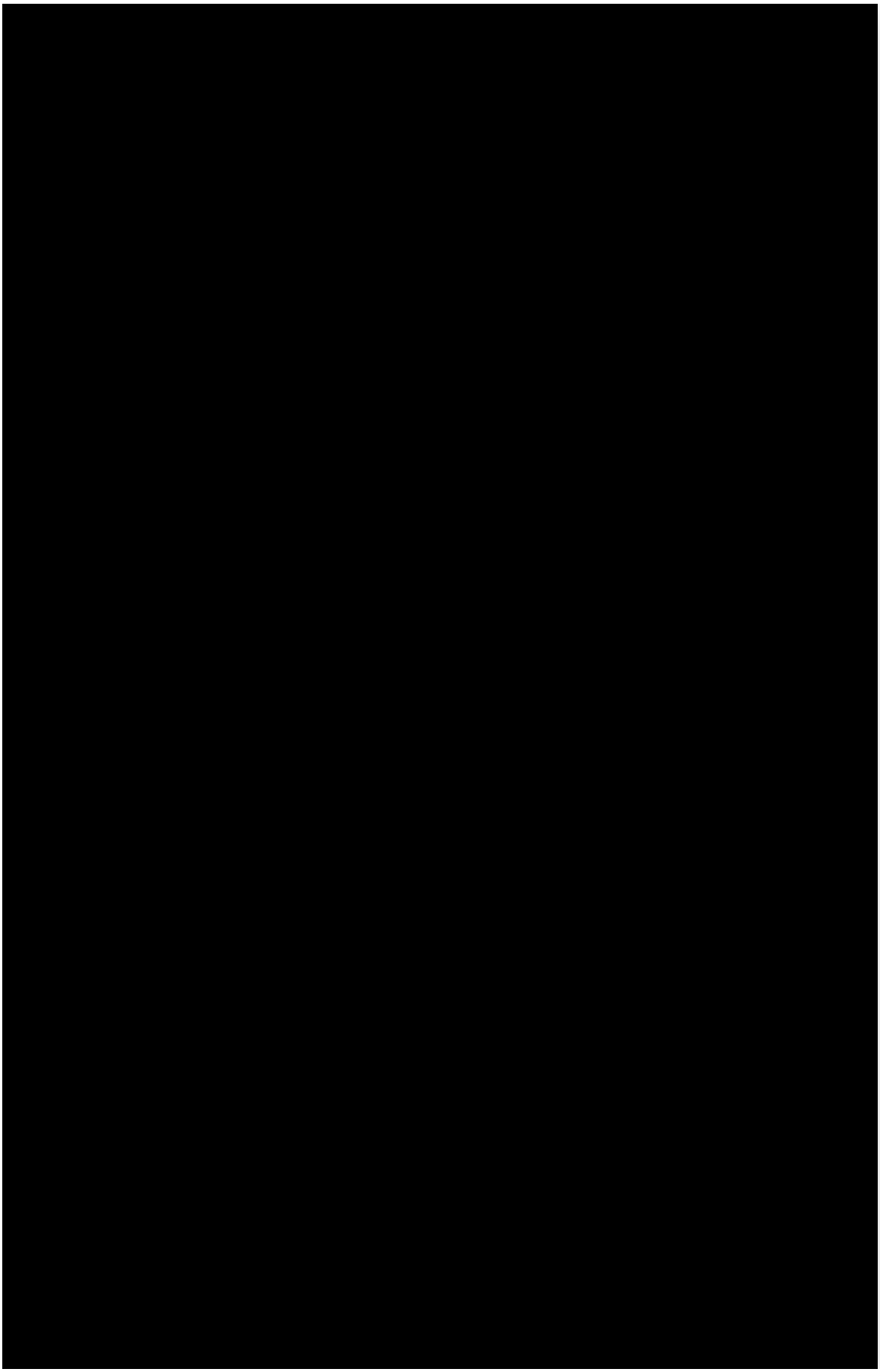
Lysis Buffer For 17.5 ml for 500 mg starting material
2.5 volume of Buffer A 6.5 ml
2.5 volume of Buffer B 6.5 ml
1.0 volume of Buffer C 2.75 ml
PVP 0.1 % 1.75 ml

Extraction I

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.

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

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 4× 15 sec bursts adding liquid nitrogen after each 15 sec grinding burst.



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

5 Incubate at RT for 30 mins mixing by inversion every 5 mins
00:30:00

6 Add 200uL Proteinase K, incubate at RT for 30 mins mixing by inversion every 5 mins
00:30:00

7 Cool on ice for 5 mins
00:05:00

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

9 Spin at 4^{O}C and 5000g for 12 mins
00:12:00

10 Transfer supernatant to fresh Falcon tube containing 17.5ml (1vol) (P/C/I) and mix by inversion for 2 mins
00:02:00

11 Spin at 4 °C and 4000g for 10 mins
00:10:00

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
00:02:00

13 Spin at 4 °C and 4000g for 10 mins
00:10:00

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

15 Incubate for 20-30mins at RT
00:20:00

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

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

18 Incubate at RT for 5-10mins
00:05:00

19 Spin at 4 °C and 10000g for 30 mins
00:30:00

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

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.

22 Spin in table top centrifuge for 5 mins at 13000g
00:05:00

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

24 Spin in table top centrifuge for 5 mins at 13000g
00:05:00

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

26 Spin in table top centrifuge for 5 mins at 13000g
00:05:00

27 Remove supernatant with pipette

28 Spin in table top centrifuge for 1 min at 13000g
00:01:00

29 Remove remaining ethanol with pipette

30 Air-dry pellet for 7 mins
00:07:00

31 Add 200uL of 10mM Tris pH9 leave at RT for 3 hours
03:00:00

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

33 leave at RT over night
16:00:00

34 Next day add another 100uL TE buffer and incubate for 1h at 28 °C with 1400rpm shaking
01:00:00

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.

Note

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

Extraction II

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

37 Elute in 10mM Tris pH8

38 Measure dsDNA concentration using BR Qubit and measure absorbance with Nanodrop

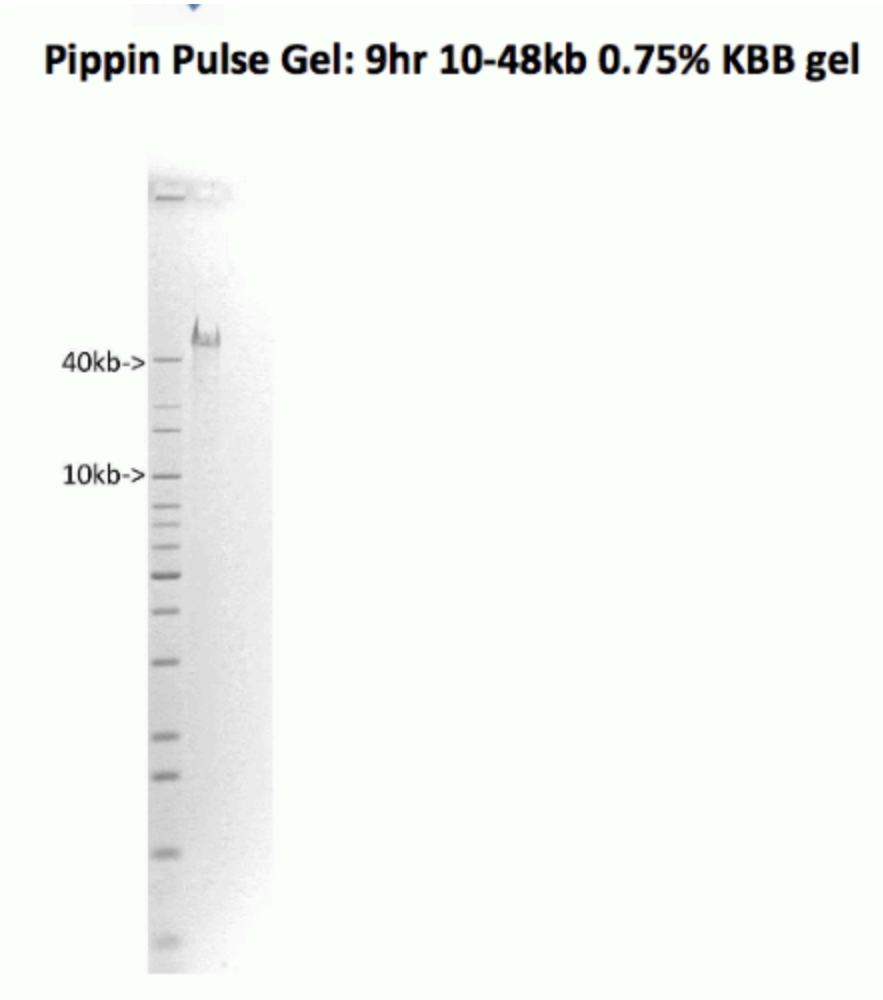
39 At this stage Qubit to Nanodrop ratios were 0.64, 260/280 1.87 and 260/230 1.37

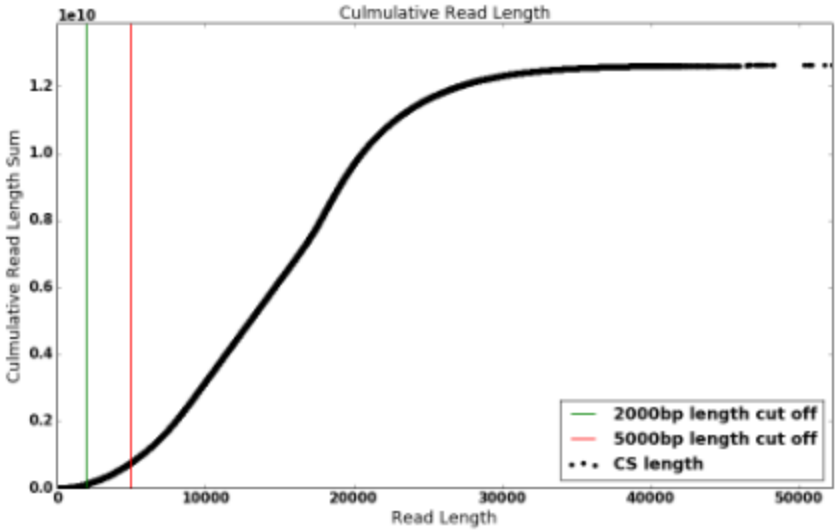
Results

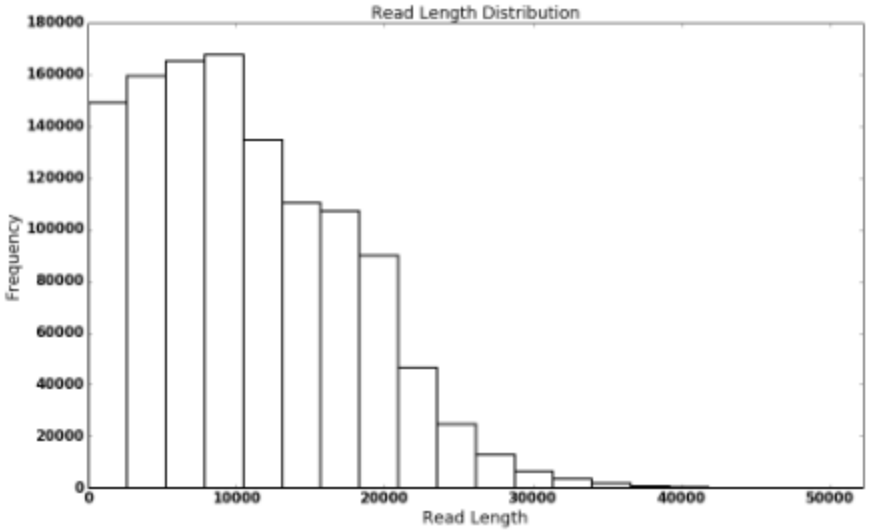
40 Samples were submitted to Ramaciotti (http://www.ramaciotti.unsw.edu.au/) sequencing centre in Sydney. Excellent personel performed quality control, prepared 15-20kb libraries and we ran 13 SMRT cells with P6 chemistry. Some summary statistics are shown below.

Expected result

Summary statistics for all 12 SMRT cells combined
********** Total sequence amount: 1.263691e+10 Number of reads: 1.181543e+06 Median read length: 9632 Mean read length: 10695




Culmulative Read Length
1e10
Culmulative Read Length Sum
Read Length
2000bp length cut off
5000bp length cut off
CS length


Read Length Distribution
Frequency
Read Length