High quality DNA from Fungi for long read sequencing e.g. PacBio, Nanopore MinION

Megan McDonald

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.

Citation: Megan McDonald High quality DNA from Fungi for long read sequencing e.g. PacBio, Nanopore MinION. **protocols.io**
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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 A 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.

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

Extraction I

Step 15.

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

Extraction I

Step 16.

Incubate at RT for 5-10mins

DURATION

00:05:00

Extraction I

Step 17.

Spin at 4 °C and 10000g for 30 mins

DURATION

00:30:00

Extraction I

Step 18.

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

Extraction I

Step 19.

Use 1mL pipette tip to transfer pellet and remaining liquid into fresh 1.7mL eppendorf tube.

If DNA is not quite pure (as is the case for some fungal DNA extractions), pellet will be brittle and will break into small pieces. Try to recover as much as possible with 1mL pipette.

If the pellet breaks apart too much, 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 2mL eppendorf tube.

Extraction I

Step 20.

Spin in table top centrifuge for 5 mins at 13000g

DURATION

00:05:00

Extraction I

Step 21.

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

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

Extraction I

Step 26.

Spin in table top centrifuge for 1 min at 13000g

DURATION

00:01:00

Extraction I

Step 27.

Remove remaining ethanol with pipette

Extraction I

Step 28.

Air-dry pellet for 2-5 mins

DURATION

00:07:00

Extraction I

Step 29.

Add 200uL (or desired final volume) of 10mM Tris pH8.5 leave at RT until dissolved

DURATION

03:00:00

Extraction I

Step 30.

Flick tube slightly for mixing. DO NOT! vortex as it shears DNA.

Extraction I

Step 31.

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

Qubit to Nanodrop ratio varies widely from fungal species to fungal species.

See next step below for comparisons before/after clean up with a silica column

Extraction I

Step 32.

Depending on Qubit/Nanodrop ratio and Nanodrop 260/280 and 230/280 ratios, further clean up sample with Genomic DNA clean-up kit with a silica column

I used ZymoResearch Genomic DNA Clean and Concentrator-10 (D4010)

Starting DNA quantification for B. sorokiniana (fungal, starting weight 200mg Freeze dried mycelia and spores) sample with clear contamination (final color of DNA extraction in Tris-HCl is yellow).

Pre Clean-up in 60 uL Total Volume:

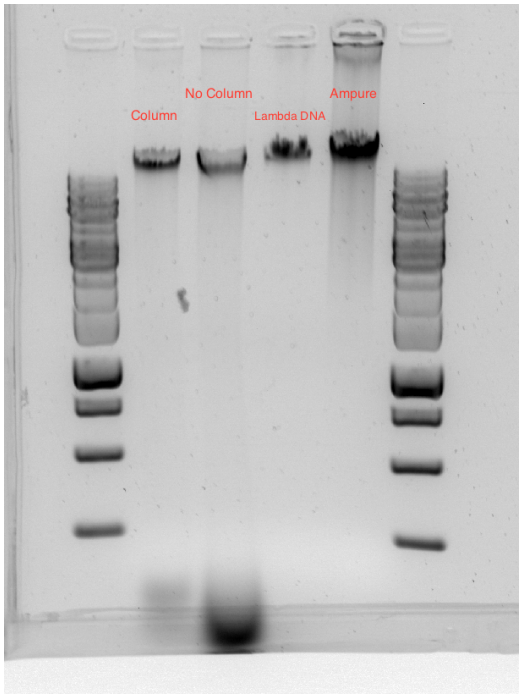
Qubit: 140 ng/uL

Nanodrop: 1800 ng/uL **260/280:**1.98 **230/280:**1.75

Post Column Clean-up in 30 uL Total Volume:

Qubit: 100ng/uL

Nanodrop: 172ng/uL **260/280:**1.91 **230/280**:2.26



Extraction II

Step 33.

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

Extraction II

Step 34.

Elute in 10mM Tris pH8

Extraction II

Step 35.

Measure dsDNA concentration using BR Qubit and measure absorbance with Nanodrop

Extraction II

Step 36.

Used 1.5 ug of starting DNA for 1D Nanopore MinION Library prep

Extraction II

Step 37.

Final DNA content of library 330ng

MinION Results

780 Mb of passed reads

Avg read length 13.3kb

Longest passed read 160kb

Total Reads (passed and failed)

1.08 Gb