High quality DNA from Fungi for long read sequencing e.g. PacBio, Nanopore MinION Version 3

Benjamin Schwessinger and 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: Benjamin Schwessinger and 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) 10 % [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 + 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.

Supernatant may/may not have a lipid layer on the top (depends on the fungus), I don't really see a difference if I take this layer or not into the phenol/choroform mix. I try to not take too much of it to make the next steps slightly easier.

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 to fresh Falcon tube. If solution remains milky, repeat P/C/I wash. If solution is clear proceed to DNA precipitation.

DNA Precipitation

Step 15.
Add 1.8mL (0.1vol) NaAc and mix by inversion

DNA Precipitation

Step 16.
Add 18mL (1vol) RT isopropanol and mix by inversion

DNA Precipitation

Step 17.
Incubate at RT for 5-10mins

DURATION
00:05:00

DNA Precipitation

Step 18.

Spin at 4 °C and 10000g for 30 mins

DURATION
00:30:00

DNA Precipitation

Step 19.

Carefully pour off supernatant till about 0.5 mL left, DNA will form a mostly translucent to white film/pellet at the bottom of the tube (colour may vary depending on your fungus).

DNA Precipitation

Step 20.

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.

DNA Precipitation

Step 21.

Spin in table top centrifuge for 5 mins at 13000g

DURATION

00:05:00

DNA Precipitation

Step 22.

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

DNA Precipitation

Step 23.

Spin in table top centrifuge for 5 mins at 13000g. Repeat wash steps (21-21).

DURATION

00:05:00

DNA Precipitation

Step 24.

Pour off ethanol and remove remaining ethanol with pipette. Spin down briefly and remove any remaining ethanol with pipette.

DNA Precipitation

Step 25.

Air-dry pellet for 2-3 mins

DURATION

00:07:00

ANNOTATIONS

Jet Beekwilder 14 Sep 2017

Hi Megan, what do you mean by 2-2 minutes? The timer says 7 minutes, did you mean 2-7 minutes?

Re-Dissolve HMW DNA

Step 26.

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

DURATION
03:00:00

Re-Dissolve HMW DNA

Step 27.

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

Quality Control

Step 28.

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

Quality Control

Step 29.

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:

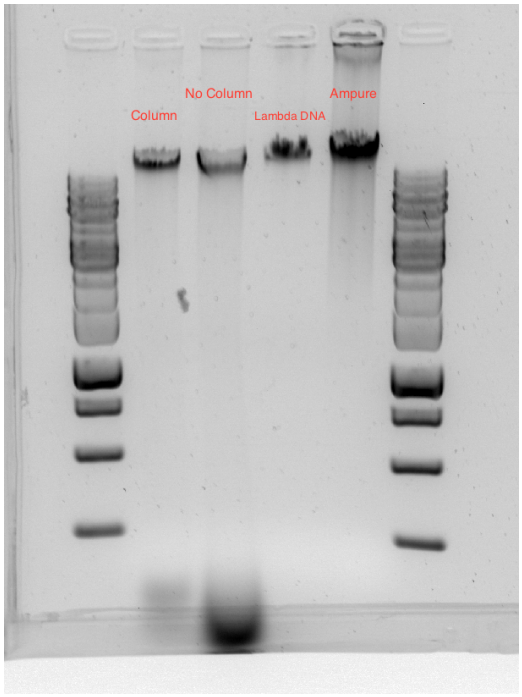
Qubit: 140 ng/uL

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



For MinIon sequencing, any visible smear indicates you have smaller DNA fragments in your sample (Visible on this gel in sample lanes 1,2 and 4). These are preferentially sequenced first on the MinIon. To increase your average read length or eliminate small reads size selection with a BluePippen (or similar is often needed). Additional clean up steps with AMPure beads can also help remove some of these small fragments.

Shearing DNA

Step 30.

NOTE: I mostly skip this step now as it introduces to many small fragments for the MinIon. Would still work well for PacBio

Cleaned up extracted DNA with Zymo Research Genomic DNA cleaner concentrator-10 Kit (D4010)

For this kit the spin speeds are too low. Spin a max speed in micro-centrifuge tube. Double check that all the liquid has gone through the column before going on to next step.

Alternative kit: Qiagen Mag Attract

Cleanup to remove small pieces

Step 31.

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

I you have low amounts of DNA you should add equal volume of beads to your sample to retain more DNA. NOTE: Adding equal volume will keep a lot more of the smaller fragments.

NOTE^2: For most fungal species I've worked with there is a contaminant that co-purifies with the AMPure beads and absorbs at 230 on the Nanodrop.

This means that my 260/230 ratios for the Nanodrop are always low (1.0-1.7). The more purification you do with AMPure the worse this ratio gets...haven't found a solution yet. MinIon or PacBio sequencing with a low 260/230 ratio still works well (you get long reads), though your total output data may be less than a sample with a better ratio.

NOTE^3:Ampure beads sometimes don't always collect nicely on the side of the tube. If this happens I remove the supernatant as best I can (SAVE IT!) and continue with the wash steps for the beads that do stick to the magnet. IN ADDITION, I add equal volume of fresh beads to my saved supernatant and purify from that solution as well. You can then mix these two samples together or run them separately on a gel to see which has less smearing (small DNA remaining).

Clean Library Ready DNA

Step 32.

Elute in 10mM Tris pH8, and measure dsDNA concentration using BR Qubit and measure absorbance with Nanodrop

MinION 1D library

Step 33.

Used 4 ug of starting DNA for 1D Nanopore MinION Library prep. Average size fragment was 20-30kb according to pulse field electrophoresis.

MinION 1D library

Step 34.

Final DNA content of library 1.8ug

MinION Results

1.8 Million reads in 24 hrs

Avg read length 11kb

ANNOTATIONS

Matt Nalley 10 Oct 2017

Hi Megan,

I'm surprised by your recovery rate (1.8 ug final library from 4 ug input DNA). Are you following the 1D protocol with all bead cleanups?

- 0.4X beads: initial DNA cleanup / removing short fragments.

- 1X beads: after nick repair

- 1X beads: after end repair

- 0.4X beads: after adapter ligation

Typically we recover 90% of DNA from 1X bead cleanups, but the 0.4X bead cleanups only recover about 50% of the DNA. Following the 1D protocol with bead cleanups as described above, with 4.0 ug input DNA we would probably have only about 800 ng in the adapted library.

Thanks!

Matt