High quality DNA from Fungi for long read sequencing e.g. PacBio, Nanopore MinION Version 4

Benjamin Schwessinger and Megan McDonald

Abstract

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

Optimized for DNA extraction from Bipolaris sorokiniana. Also tested on Parastaognospora nodorum, Zymoseptoria tritici, wheat stripe rust, barley stripe rust and Pyrenophora tritici-repentis

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.

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.

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:

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Reagents required

BUFFER A: 0.35 M sorbitol

0.1 M Tris-HCl

5 mM EDTA, pH 8

autoclave to sterilize

BUFFER B: 0.2 M Tris-HCl

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)

Polyvinylpyrrolidone (10000 MW) 10% [w/v] (Sigma PVP10)

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 A or T1 (1000 U/ml, Thermo Fisher EN0541)

Proteinase K (800U/ml, NEB P81072)

AMPure beads from Beckman

Lysis Buffer For 14 ml for 500 mg starting material

2.5 volume of Buffer A 5 mL

2.5 volume of Buffer B 5 mL

1.0 volume of Buffer C 2 mL

PVP 40 10% 1mL

PVP 10 10% 1mL

Protocol

Extraction I

Step 1.

Make lysis buffer by mixing buffer A+B+C+PVP10+PVP40 in 50mL Falcon tubes.
All following steps are based on 14 mL 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 2.8 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 mL (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

ANNOTATIONS

Elspeth Ransom 13 Apr 2018

A quick question about this step. I get a thick sticky layer at the top (I assume the lipid layer?), then liquid below which I take but I am not seeing a pellet. I am not sure if I am taking the correct layer. Is this what you see?

Many thanks

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 ml (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 50 mL Falcon tube. If solution remains milky, repeat P/C/I wash. If solution is clear proceed to DNA precipitation.

Usually I am able to transfer 15 mL of the supernatant.

DNA Precipitation

Step 15.

Add 1.5mL (0.1vol) NaAc and mix by inversion

DNA Precipitation

Step 16.

Add 15mL (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 8000g for 30 mins

DURATION

00:30:00

DNA Precipitation

Step 19.

Carefully pour off supernatant.

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.

Wash pellet in 5mL of fresh 70% EtOH. Gently tap tube to dislodge the pellet from the side to get a thorough wash.

Spin 3000-5000g for 3-5 min to stick pellet back to the side of the falcon tube.

DNA Precipitation

Step 21.

Pour off EtOH wash untill about 0.5 mL remaining. Gently dislodge pellet with 1mL pipette tip and pour pellet with remaining 70% EtOH into fresh 1.5mL 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 22.

Spin in table top centrifuge for 5 mins at 13000g

DURATION

00:05:00

DNA Precipitation

Step 23.

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

DNA Precipitation

Step 24.

Spin in table top centrifuge for 5 mins at 13000g. Repeat wash steps (21-24).

DURATION

00:05:00

DNA Precipitation

Step 25.

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

DNA Precipitation

Step 26.

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 27.

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 28.

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

Quality Control

Step 29.

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 30.

Measure DNA concentration with the Qubit and Nanodrop.

The closer your nanodrop measurement is to your Qubit the better.

Typical values after my DNA extractions are:

B. sorokiniana CS27

Sample	Volume	Qubit ng/uL	Nano ng/uL	260/230	260/280
CS27	200 uL	65	1389	1.94	1.92
CS27-2	200uL	84	1800	1.92.	1.93

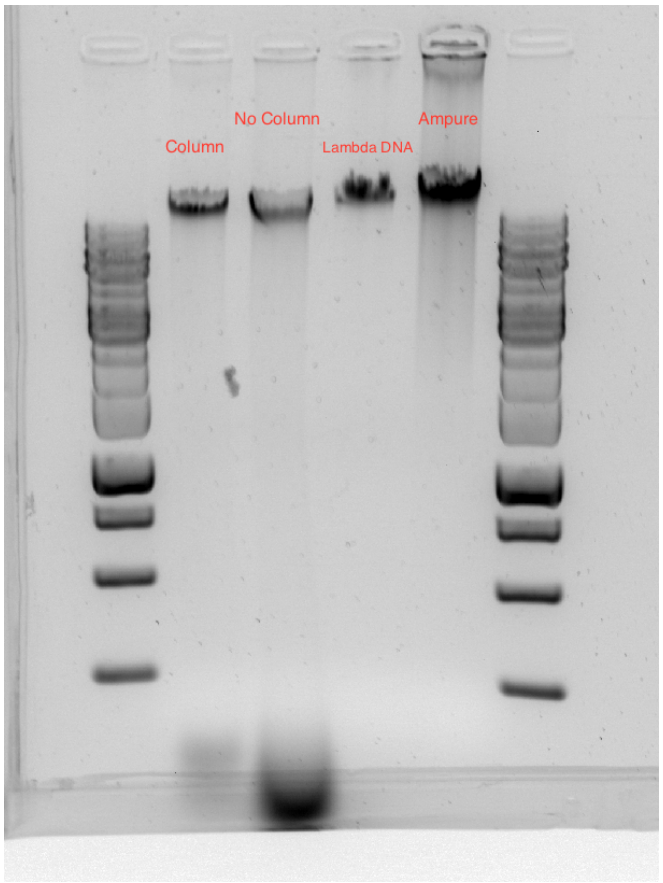
Cleanup to remove small pieces

Step 31.

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

Repeat AMPure bead clean-up at LEAST TWICE before starting MinIon Library Prep.

Run agarose gel to check for remaining RNA, small fragmets or smears. You want your sample to look like Lane 4 (Ampure) where there is NO visible smear on the lower parts of the gel.



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

Cleanup to remove small pieces

Step 32.

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.

Example of Nanodrop values decreasing with every AMPure clean up:

Sample	Volume	Qubit ng/uL	Nano ng/uL	260/230	260/280
CS27	200 uL	65	1389	1.94	1.92
CS27-amp1	100 uL	114	291	1.74	1.09
CS27-amp2	50 uL	102	305	1.68	0.92

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!). Then BREIFLY spin the tube to help the beads pellet. (The more you spin the more you will shear your DNA). Remove as much as the supernatant as you can. Then proceed to next step. During 70% EtOH wash with beads, gently rotate the 1.5mL tube 360º on the magnet. The beads will slowly follow as you rotate. This helps get a better wash.

If your final ellution doesn't contain DNA, you can recover it from the SAVED supernate from the very first step. To recover add equal volume of beads to this saved supernatant and proceed with normal protocol.

ANNOTATIONS

Caio Leal-Dutra 30 May 2018

Hi Megan,

Do you have any update in this 230nm contamination issue?

Thanks,

Caio

Clean Library Ready DNA

Step 33.

Elute in 50 uL 10mM Tris pH8, and measure dsDNA concentration using BR Qubit and measure absorbance with Nanodrop

MinION 1D library

Step 34.

Stats shown below are for sample **CS27-amp2**

Used 5 ug of starting DNA for 1D Nanopore MinION Library prep.

Remaining DNA after FFPE repair: 3.75 ug

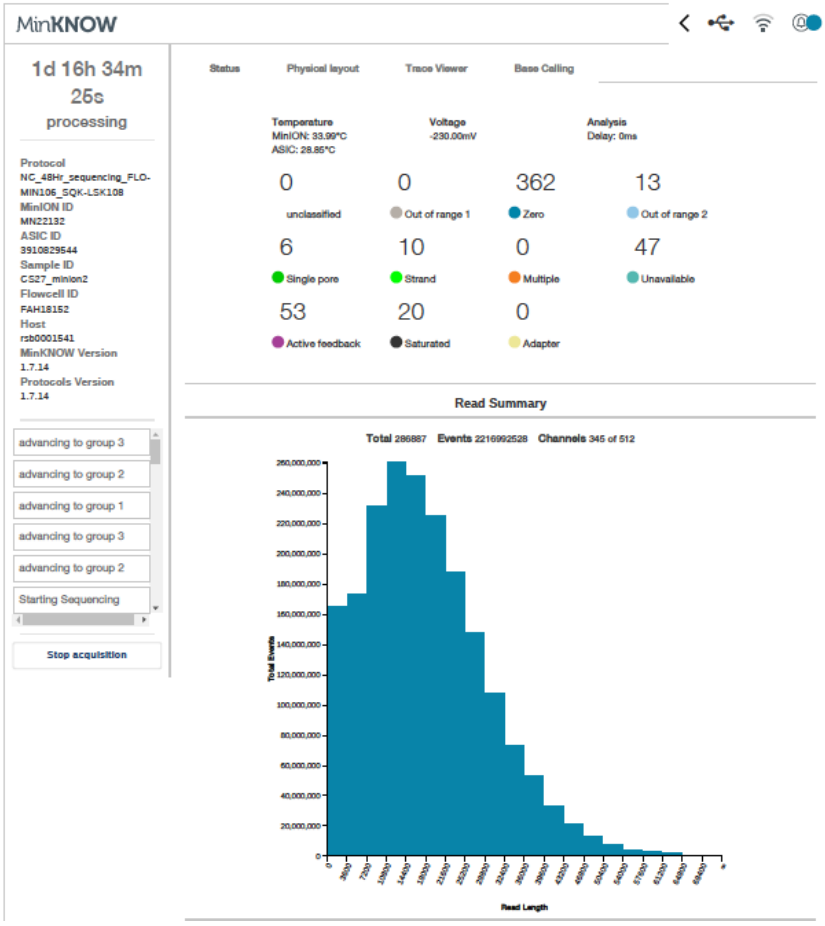
Remaining DNA after daTailing 2.7 ug

Remaining DNA after Adapter Lig 1 ug

MinION 1D library

Step 35.

MinIon Results after 40 hrs



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