

# High quality DNA from Fungi for long read sequencing e.g. PacBio, Nanopore MinION Version 2

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 grateful for critical suggestion from the following scientists.  
Dr. Claire Anderson, Dr. Andrii 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:Isoamylalcohol 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.**

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.**

CarefullPour 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. Repeat wash steps (21-21).

 DURATION

00:05:00

#### Extraction I

##### Step 23.

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

#### Extraction I

##### Step 24.

Air-dry pellet for 2-2 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?

#### Extraction I

##### Step 25.

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

 DURATION

03:00:00

#### Extraction I

##### Step 26.

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

## Extraction I

### Step 27.

Measure dsDNA concentration using BR Qubit and measure absorbance with Nanodrop. This might be also a good step to assess DNA quality by running a 0.8% TBE agarose gel with 500ng dsDNA and a lambda-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 28.

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:

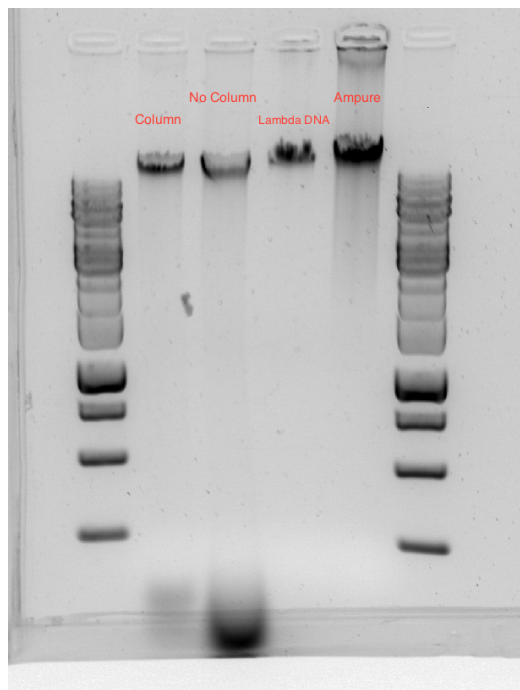
Qubit: 140 ng/uL

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**



## Shearing DNA

### Step 29.

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 30.

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

## Clean Library Ready DNA

### Step 31.

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

## MinION 1D library

### Step 32.



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 33.**

Final DNA content of library 1.8ug

#### MinION Results

1.8 Million reads in 24 hrs

Avg read length 11kb

Basecalling will take forever...need to update