High purity, high molecular weight DNA extraction from rust spores via CTAB based DNA precipitation for long read sequencing

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Abstract

Nanopore sequencing (Oxford Nanopore Technologies MinION instrument) requires high quality, high molecular weight DNA (HMW) to produce long sequence reads. Extracting high purity, HMW DNA is a difficult challenge, especially for a biotrophic rust fungus. This is because the biomass for DNA extraction is restricted to spores which are physically tough and rich in complex polysaccharides and lipids. When DNA extracted from spores precipitated with ethanol or isopropanol, many other impurities like polysaccharides and lipids get coprecipitated along with DNA which renders DNA purity unsuitable for Nanopore sequencing. These impurities cannot be removed with any subsequent DNA purification with either paramagnetic beads (SPRI) or re-precipitation with ethanol and isopropanol. These impurities always co-precipitated with the DNA which absorbs light at 230 nm. In an attempt to improve purity, I tried precipitating DNA with cationic detergent CTAB (Hexadecyl-Trimethyl-Ammonium Bromide) which has been used to precipitate DNA in the past but not employed extensively. CTAB is a cationic surfactant which selectively complex with DNA and precipitates it out of the solution in the presence low salt concentration (0.4M NaCl) while leaving other impurities.

Unlike isopropanol or ethanol precipitated DNA which looks highly viscous, the CTAB precipitated DNA lack similar viscosity and therefore indicative less or no impurities. Moreover, with CTAB precipitation I get ~ 20-25 ug crude DNA per 100 mg of spores which is almost double the amount I get with isopropanol and ethanol precipitation. The crude DNA once washed with 1 V homemade SPRI beads solution, gives a 260/280 ratio ~1.8-2 and 260/230 2.0-2.2.

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Guidelines

I adopted this protocol from “Modified low-salt CTAB extraction of high-quality DNA from contaminant-rich tissues” by Jean-René Arseneau, Royce Steeves, and Mark Laflamme.

https://onlinelibrary.wiley.com/doi/full/10.1111/1755-0998.12616

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Note: We started DNA extraction and optimization with the aim to get pure DNA from the rust spores which meet all the purity standard needed for Nanopore sequencing. This protocol gives the DNA that meets all the purity standards like the 260/280 and 260/230 ratios and the Nanodrop and Qubit concentration 1:1, **despite this we could not get a good nanopore run out of this DNA**.

This could be due to some contaminants are getting copurified with the DNA which interferes with the nanopore sequencing. These contaminants are invisible to the Nanodrop light spectrum.

This probably might just be an issue with DNA extracted from rust spores and for other species, the protocol might just work fine.

We are planning to purify this DNA with low melting point (LMP) agarose plugs to test if the sequencing run improves.

We would update the protocol as and when we succeed in getting an awesome run!

Materials

- CTAB by Contributed by users
- 0.5 M EDTA by Contributed by users
  5 M Sodium chloride (NaCl) S5150-1L by Sigma Aldrich
- 1 M Tris-HCl pH 8.0 by Contributed by users
- 10 % PVP (M. Wt. 10K) w/v by Contributed by users
- Ethanol 70% by Contributed by users
- Chloroform: Isoamyl Alcohol (24:1) by Contributed by users
- Autoclave acid washed Sand by Contributed by users
- Homemade SPRI beads solution for DNA clean-up by Contributed by users
- RNAas T (1000 U/ml, Thermo Fisher EN0541 by Contributed by users
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Protocol

Preprations:

Step 1.

Prepare Buffer Solutions:

CTAB lysis buffer:

Tris =100 mM
EDTA=20 mM
CTAB 2% w/v
NaCl 1.2M
PVP 1 % (M. Wt. 10K) 1% v/v

CTAB precipitation buffer:

TrisHCl=100 mM
EDTA=20 mM
CTAB 2% w/v

Preprations:
Step 2.

Set a water bath at 60 °C and assemble the extraction buffer by adding 1 % PVP (v/v) into the CTAB lysis buffer and keep the buffer in the water bath to pre-warm.

Preprations:
Step 3.

Pre-chill mortar, pestle, and sand: Weigh 200 mg acid washed autoclaved sand per 100 mg of tissue, add it to clean sterile mortar, pure liquid nitrogen and let it dissipate.

Preprations:
Step 4.

Meanwhile, weigh 100 mg spores and add to the mortar as the last drop of the liquid N2 dissipate. Mix tissue with sand using the pestle before adding more liquid N2.

Crude DNA extraction:
Step 5.

Grinding the spores/tissue: Grind spores with liquid nitrogen 3-4 time for 10 sec adding liquid nitrogen each time. Do not exert an excessive pressure while grinding the spores as this may sheer your DNA.

Crude DNA extraction:
Step 6.

Aliquot 5 mL CTAB lysis buffer into 50 mL Falcon tubes and add tissue samples. Put the tube on a tube rotator for 10 min and once the sample homogenized with the buffer add 50 µL of Proteinase K (stock conc. = 20 mg/mL) to each sample and incubated for 1.5 hours at room temperature on a tube rotator.

Crude DNA extraction:
Step 7.

Add one volume (5mL) of Chloroform: Isoamyl alcohol (24:1) to each tube. Mix well by rotation for 15 min. Centrifuge at room temperature for 15 minutes at 5000g.

Crude DNA extraction:

Step 8.

Transfer the aqueous phase to a new 50 mL tube. Add 5 ul RNase T (1000 u/ ul) and incubate on RT for 30 min on the rotator.

Crude DNA extraction:

Step 9.

Repeat the CI extraction, add I volume (5mL) of Chloroform: Isoamyl alcohol (24:1) to each tube. Mix well until the upper aqueous phase becomes cloudy. Rotate For 15 min then centrifuged at room temperature for 15 minutes at 5000g.

Crude DNA extraction:

Step 10.

DNA precipitation: To precipitate DNA with CTAB buffer, transfer the aqueous phase to a new 50 mL tube and add 2 volumes of CTAB precipitation buffer to the aqueous phase. Mix well by inverting the tubes and place in a rotating hybridization oven or any other temperature control rotator set at 55 °C for 30 until white crystals (Fig. 1) can be observed floating inside the tubes.

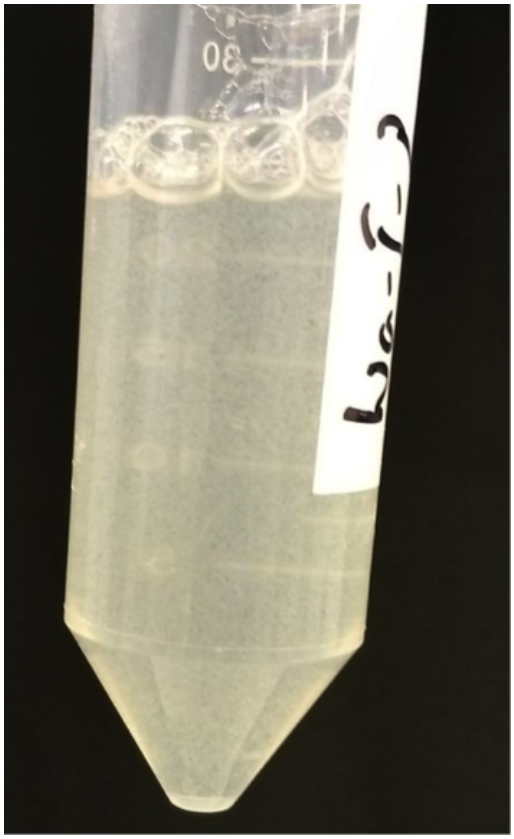


Fig. 1: CTAB-DNA complex white crystals floating in the solution

TEMPERATURE

60 °C Additional info:

Crude DNA extraction:

Step 11.

Centrifuge at 16,000g for 5 minutes at RT to precipitate the CTAB-DNA complex. Carefully discard the supernatant without disturbing the pellet (Fig. 2).

Fig. 2: DTAB-DNA complex pellet

Crude DNA extraction:

Step 12.

Add 5 ml freshly prepared 70 % ETOH, invert the tube multiple time and let soak for 15 minutes at RT to remove excess salts and CTAB.

Crude DNA extraction:

Step 13.

Centrifuge again at 16,000g for 5 minutes and carefully discard the supernatant without disturbing the pellet.

Crude DNA extraction:

Step 14.

Air dry the pellet for 10-15 min or until ethanol evaporate and dissolve the pellet in 200 ul TE buffer by taping the tube with a finger and transfer into a 1.5 ml tube.

Purification:

Step 15.

Purification of crude DNA with SPRI paramagnetic beads: To remove any residual impurities, short oligonucleotides and RNA, I wash crude DNA with first 2 volume and then 1-0.9V of the homemade SPRI beads solution. This help in getting rid off most impurities and short DNA fragments and give recovery of 60-70 percent high quality high molecular weight DNA.

For detailed purification protocol, please refer to the SPRI based DNA purification protocol we published on protocol.io
recently, https://www.protocols.io/view/dna-size-selection-3-4kb-and-purification-of-dna-u-n7hdhj6

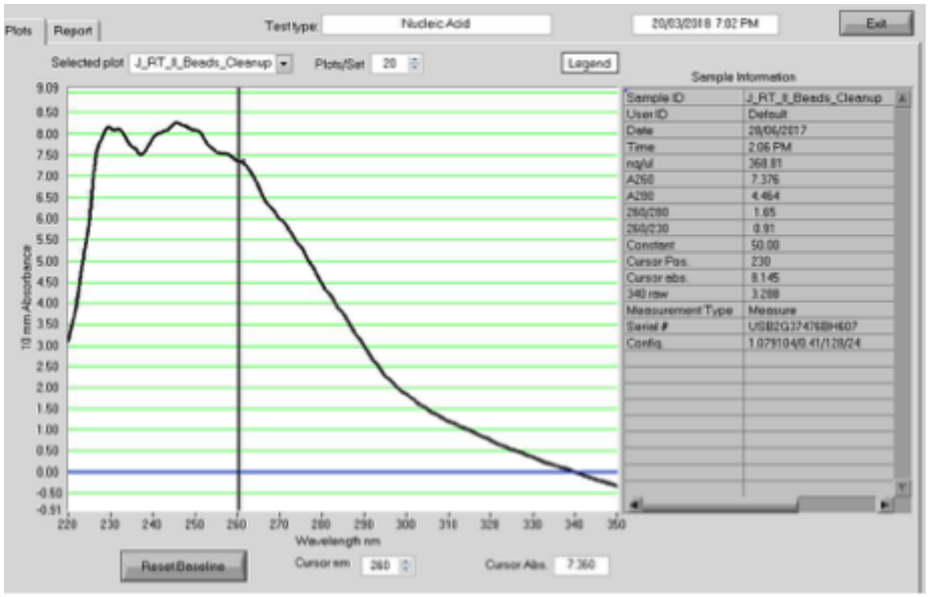
DNA quantification and DNA quality evaluation:

Step 16.

DNA quantification and DNA quality evaluation: Measure DNA with a fluorometric assay such as Qubit (Invitrogen) or Quantus (Promega), then measure with a spectrophotometer like Nanodrop and observer the 260/280, 260/230 ratios and concentration. Ideally, the pure DNA should have a 260/280, 1.8 and, 260/230, 2.0 and the concentration measured by both fluorometric assay and spectrophotometer 1:1 or 1:1.2. The CTAB precipitate, 1 volume SPRI beads wash DNA meet all the purity standard (Table 1) and gives a NanoDrop absorbance spectrum typical of pure DNA (Fig. 3)

A

B



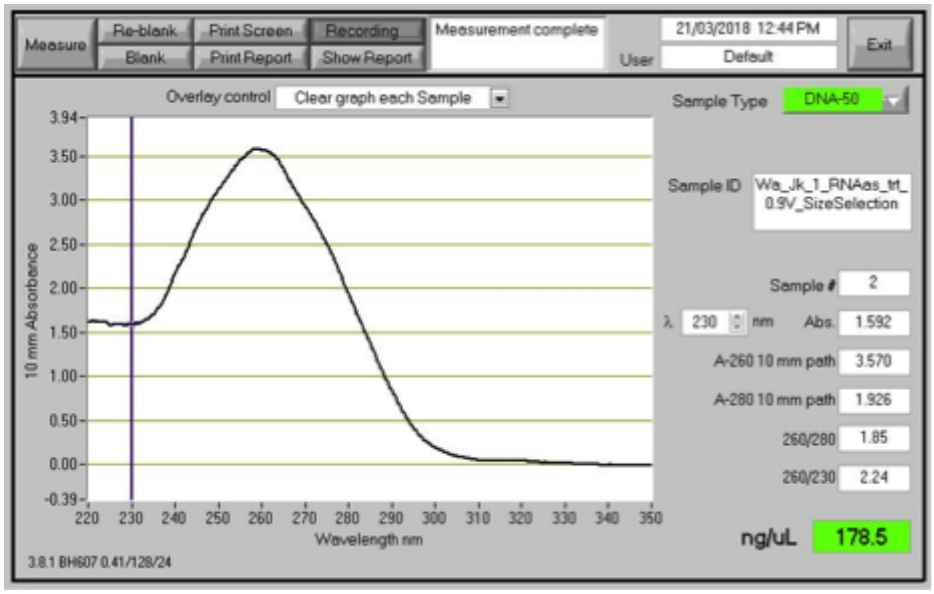


Fig. 3: NanoDrop spectrophotometer absorbance spectra of DNA extracted with (A) 1V isopropanol precipitation (B) 2V CTAB buffer precipitation.

DNA quantification and DNA quality evaluation:

Step 17.

Table 1: DNA concentration measured with NanoDrop and Quantus (Promega)

Sample	Precipitation method used	Quantus concentration (ng/ul)	NanoDrop concentration (ng/ul)	260/280	280/230
Rust_spores_1	2V CTAB precipitation buffer	158	178	1.88	2.2
Rust_spores_2	2V CTAB precipitation buffer	216	224	1.86	1.99

DNA quantification and DNA quality evaluation:

Step 18.

Fig. 4: Visual inspection of the CTAB precipitate, 1V SPRI beads purified DAN on 1% TBE agarose gel. The lane 1 from the left is NEB HindIII cut lambda DNA and lane 2-4 are three independent DNA extractions from rust spores.

DNA quantification and DNA quality evaluation:

Step 19.

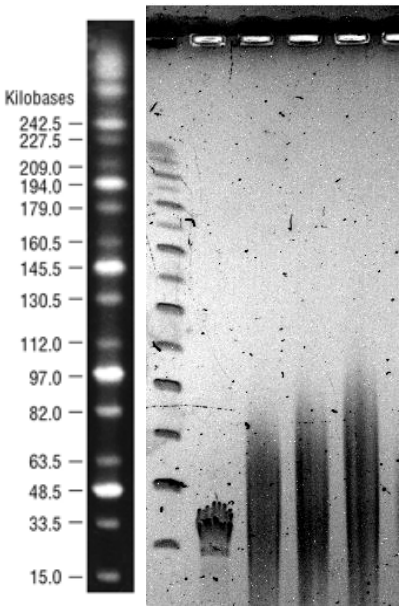


Fig. 5: Pulsed Field Gel electrophoresis of CTAB precipitation 1V beads washed DNA. The lane 1 and 2 from the left are NEB mid-range PFGE marker and HindIII cut lambda DNA respectively. The lane 3-5 from left are three independent DNA extractions from rust spores.