Multi-step high purity high molecular weight DNA extraction protocol from challenging fungal tissues

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Abstract

This protocol is based on the fact that we struggled for a long time to obtain high purity DNA from the fungal material, especially from several rust species. We finally thought we made it when we obtained DNA with perfect QC measures using a CTAB based DNA precipitation. Yet we still got low yields caused by high amount of 'active feedback' during the sequencing run. We and many others had similar observations that awesome looking DNA doesn't sequence well (https://www.protocols.io/groups/awesome-DNA-from-all-kingdoms-of-life/discussions/awesome-dna-purity-measures-but-quickly-dying-pores).

In this protocol, we combined several ideas we accumulated over the last 1.5 years since we started working on HMW DNA extractions for Nanopore sequencing.

-> Different precipitants have different affinities for different contaminants.

-> Some contaminants may have a higher affinity and lower solubility in NaCl/PEG/SPRI 'clean' up steps.

-> Adding enzyme cocktails during the extraction may help to get rid of some contaminants.

We combined all these three steps in the current protocol as combinatorial testing is currently cost prohibitive. It may well be that one of these steps is already enough.

These ideas are laid out in more detail below and in publication soon to come.

Our general recommendation is to test different buffer conditions and precipitants and if necessary combine them in a sequential manner. We hypothesize that different precipitants, e.g. NaCl/PEG, isopropanol, ethanol, or CTAB, display varying affinities for precipitating different contaminants. By applying them in a sequential manner it may be possible to obtain clean DNA via preferential precipitation of DNA over contaminants. In addition, in this newly developed protocol, we add enzyme mixes to the extraction buffer containing pectinases and cellulases reducing the amount of co-purifying contaminants from the fungal tissue. In case of other tissue types, different enzymes may have to be tested. It is important to add these enzymes during the extraction and not apply them to the final DNA suspension as most are not completely pure enzyme preparations and contain traces of DNAase activity that degrades the DNA when applied in simple solutions like TE buffer.

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We (see above) and many others have reported that NaCl/PEG-SPRI bead solutions are not always ideally suited to clean up DNA as contaminants simply co-precipitate. Following a similar logic of preferential precipitation, We hypothesize that is possible to first precipitate contaminants onto SPRI beads at low NaCl/PEG concentrations when HMW DNA stays in solution. In a subsequent step, DNA can be precipitated out of the remaining supernatant by increasing NaCl/PEG concentration adding more of the initial NaCl/PEG-SPRI beads solution. Contaminants with higher affinity to SPRI beads and lower solubility than DNA can thereby be removed from the solution.

It is important to mention that we have had DNA preparations that fulfilled all our recommended quality control criteria but did not sequence well on the MinION. This was likely caused by 'invisible' contaminants. However, applying a combination of the above-suggested approaches enabled us to overcome this problem with our latest protocol.

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Guidelines

This protocol is a significantly extended version and based on "Modified low-salt CTAB extraction of high-quality DNA from contaminant-rich tissues" by Jean-René Arseneau *et.al.*

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Materials

- RNase T1 EN0541 by Thermo Scientific
- Isopropanol by Contributed by users
- Proteinase K EO0491 by Thermo Fisher Scientific
- 3 M Sodium acetate, pH 5.2 by Contributed by users
- 0.5 M EDTA Stock Solution (adjusted to pH 8.0 with NaOH) by Contributed by users
- 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
- Autoclave acid washed Sand by Contributed by users
- 10 mM Tris-HCL pH 8.0 by Contributed by users
- 5 M NaCl by Contributed by users
- Chloroform:Isoamyl alcohol 24:1 C0549 by Sigma
- SPRI beads solution View by Contributed by users

CELLULASE ONOZUKA R-10 View by Contributed by users

Pectinase from Aspergillus niger View by Sigma

Macerozyme R-10 View by Contributed by users

Protocol

Buffers composition

Step 1.

CTAB lysis buffer pH 8:

Tris =100 mM

EDTA=20 mM

CTAB 2% w/v

NaCl 1.2M

CTAB precipitation buffer pH 8:

TrisHCl=100 mM

EDTA=20 mM

CTAB 2% w/v

Assembling the lysis buffer

Step 2.

Take 5 ml CTAB lysis buffer per 100 mg spore extraction and add 1.0 % PVP (v/v), 1.0 % Cellulase (w/v), 1.0 % Pectinases (v/v) and 1.0% Macerozyme (w/v).

Prechill mortar, pestle, and sand

Step 3.

Weigh 2 g acid washed autoclaved sand per 100 mg of tissue and add to a clean sterile mortar, carefully and slowly pure liquid nitrogen and let it dissipate twice.

Step 4.

Meanwhile, weigh 100 mg spores. Add spores to the mortar once the last drop of the liquid N2 is gone and no N2 is left in the mortar. Mix spores with sand using the pestle and add more liquid N2 once spores and sand are properly mixed quickly.

Grinding the spores

Step 5.

Grind spores 3-4 times for 10-15 sec bursts adding liquid nitrogen each time. Do not exert excessive pressure while grinding the spores as this may shear the DNA.

Step 6.

Transfer the ground spore to the 50 mL Falcon tube containing the assembled CTAB lysis buffer (see step 1).

DNA extraction

Step 7.

Add 50 µL of Proteinase K (stock conc. = 20 mg/mL) and incubated for 1.5 hours at room temperature on a rotator with 30 rpm.

Step 8.

Add one volume (5 mL) of Chloroform: Isoamyl alcohol (24:1). Mix well by inverting the tube for 10-15 times and centrifuge for 10 minutes at 5000g at room temperature.

Step 9.

Carefully 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 a tube rotator with 30 rpm.

Step 10.

Repeat the C:I extraction. Add I volume (5 mL) of Chloroform: Isoamyl alcohol (24:1) to each tube. Mix well by inverting the tube for 100 times and centrifuge for 10 minutes at 5000g at room temperature.

DNA precipitation

Step 11.

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

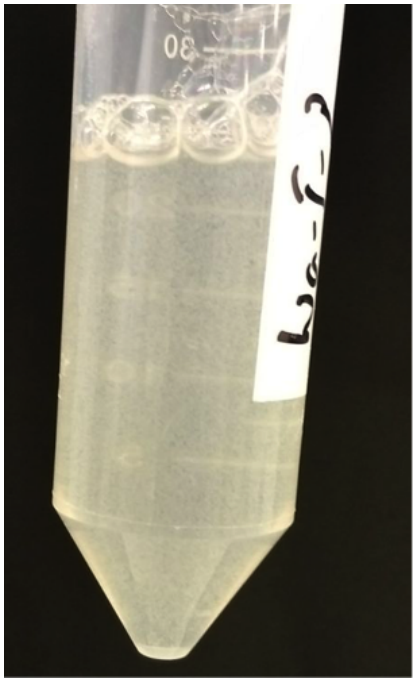

Fig. 1: CTAB-DNA complex white crystals floating in the solution.

Step 12.

Centrifuge at 16,000g for 5 minutes at RT to pellet crystals. The pellet can be is quite fragile, depending on the sample and amount of the DNA, so care must be taken while pipetting off the supernatant (Fig. 2).

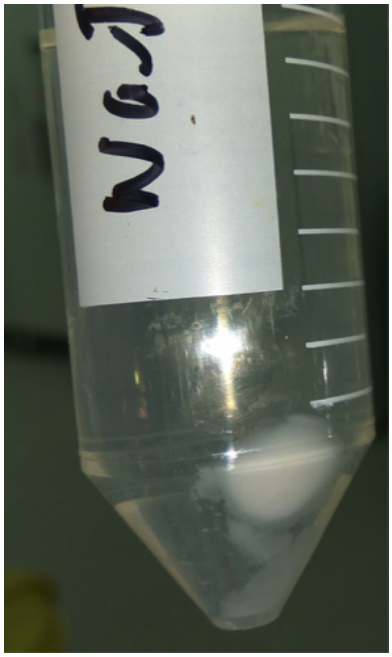

Fig. 2: CTAB-DNA complex pellet

Step 13.

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

Step 14.

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

Step 15.

Air dry the pellet for 10-15 min or until ethanol evaporates and dissolve the pellet in 500 ul 10 mM Tris (pH-8.0) and transfer into a 2 ml tube.

I refer to the DNA obtained at this stage of the protocol as crude DNA.

Crude DNA purification using RNAse, proteinase K, C:I and isopropanol precipitation

Step 16.

Adding RNase in the lysis buffer doesn't really help in completely removing RNAs. This could be because of the complexity of the lysis buffer which inhibits the RNase activity. To get rid of RNAs and other carried over impurities like histones proteins which may still bound with the DNA, I include RNase and an additional Proteinase K treatment.

Step 17.

Add 5 ul RNase T (100U/ul) in the 500 ul crude DNA and incubated at room temperature on a horizontal shaker at 400rpm for 1 hour.

Step 18.

Followed by add 10 ul Proteinase K and incubated at room temperature on a horizontal shaker at 400rpm for 1 hour.

Step 19.

Add the equal volume chloroform: isoamyl alcohol (24:1) and mix 100 times slowly by hand.

Step 20.

Centrifuge at 5000 g for 10 min and carefully take the upper aqueous phase into a fresh 15 ml falcon tube.

Step 21.

To recover DNA from the aqueous phase, add 0.1 volume of 3 M sodium acetate (pH-5.2) into the aqueous phase and mix well

Step 22.

Now precipitate the DNA with an equal volume of isopropanol and pellet down the DNA by centrifugation at 5000 g for 10 min.

Step 23.

Pipette off the supernatant and wash the pellet with 70% freshly prepared ethanol twice.

Step 24.

After the second wash spins down briefly and removes all remaining ethanol with a pipette.

Step 25.

Add 200 ul 10 mM Tris (pH-8) to the pellet and dissolve overnight.

2 Volume SPRI beads Wash

Step 26.

Due to residual RNAs, small oligos and carry over salts, DNA concentration measured at this stage are potentially overestimated by both Qubit and NanoDrop. Washing DNA with 2 V of SPRI magnetic beads solution helps to remove these residual RNAs, small oligos and nucleotides result in intact double-stranded DNA molecules. After this SPRI beads wash the concentration measured by Qubit and Nanodrop should be close to 1:1. The recovery of DNA at this step will depend on the purity of the input sample prep. Typically experience % recovery are between 50-80%.

The SPRI beads solution I used here is prepared in the lab and the recipe can be found with this link.

DNA quantification and DNA quality evaluation

Step 27.

To measure the intact double-stranded DNA concentration, use a fluorometric assay kit such as Qubit (Invitrogen) or Quantus (Promega). To access purity use a UV spectrophotometer like Nanodrop and observe the 260/280, 260/230 ratios and concentration. Ideally, pure DNA should have a 260/280, 1.8 and, 260/230, 2.0-2.2 and the concentration measured by both fluorometric assay and spectrophotometer should be 1:1 to 1:1.5. The CTAB precipitated, 2 V SPRI beads wash DNA meets these purity standards (Table 1) and gives a NanoDrop absorbance spectrum typical of pure DNA. To visually inspect the DNA, run a regular 0.8 % TBE agarose gel and observe the DNA sample for any residual RNA and other contamination, moslty found at the bottom of the gel. Load 50-100 ng dsDNA along with 100 ng Hind-III cut lambda DNA ladder. To access the DNA molecules size distribution, run a pulse field gel.

Comparison of ethanol/isopropanol and CATB precipitated DNA:

Table: 1

Sample	Precipitation method used	Quantus concentration (ng/ul)	NanoDrop Concentration (ng/ul)	260/280	280/230

Sample1	2V CTAB precipitation buffer	158	178	1.88	2.2
Sample2	2V CTAB precipitation buffer	216	224	1.86	1.99

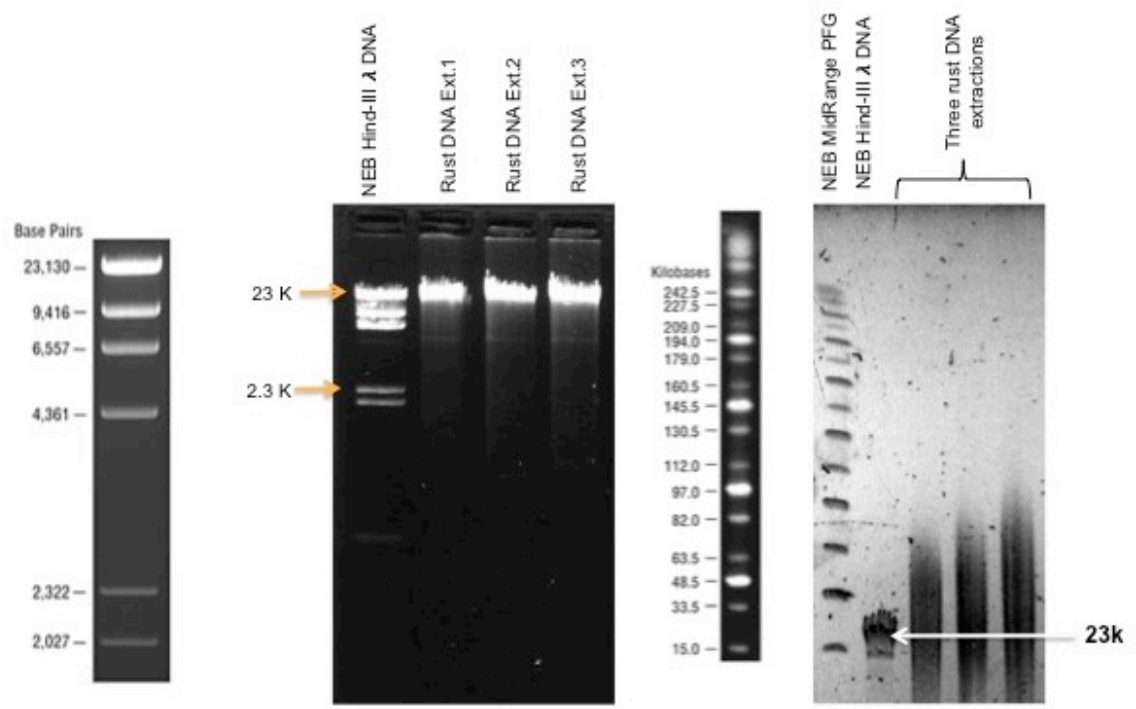

Fig. 3: RNase treated, 2V SPRI beads purified rust DNA from three independent rust DNA extractions using CTAB for precipitation run on a regular 1% TBE agarose gel (left) and on a pulsed-field (right).

Purification of crude DNA with low volume SPRI beads before sequencing

Step 28.

I wash rust DNA with 0.4-0.6V homemade SPRI beads solution before making the library for nanopore sequencing. We believe this step helps in removing some contaminant that binds with high affinity to SPRI beads at a PEG/NaCl concentration where most DNA stays in solution (Fig. 4). At this sample to bead volume ratio (1:0.6) 90-95 % DNA remain in the solution. This DNA can be recovered by adding more PEG/NaCl-SPRI bead solution to the supernatant of the 0.4-0.6 V treatment. The final beads volume ratio should be 1: 2.0.

This step may remove some of your ultra long DNA fragments (as shown in Fig. 4). Yet we did not see a significant decrease in read N50 sequencing this sample when compared to other DNA extraction methods.

Step 29.

Add 0.6 V of PEG/NaCl-SPRI bead solution (as described here) to the DNA and mix well by horizontal shaking or flicking the tube gently for 5 mins.

Step 30.

Put the DNA beads mix to a magnet block and allow beads to settle at the magnet wall.

Step 31.

Take the **supernatant** in a fresh tube and add additional SPRI beads solution up to a final DNA: beads ratio of 1:2. mix well by keeping the tube on a horizontal rotator for 5 min.

Step 32.

Keep the tube on a magnet block and let the beads settle down on the wall.

Step 33.

Remove the supernatant and wash the pellet with freshly prepared 70% ethanol twice and air dry the pellet for 30-40 seconds.

Quantification and QC analysis

Step 34.

Dissolve the pellet in 100 ul 10 mM Tris pH-8. Quantify the DNA once again with both Qubit and NanoDrop to know the absolute DNA concentration and the purity of your sample. You should get 1:1 DNA concentration measured by both NanoDrop and Qubit and ideal 260/280 (1.8) and 260/230 (2.2) ratio.

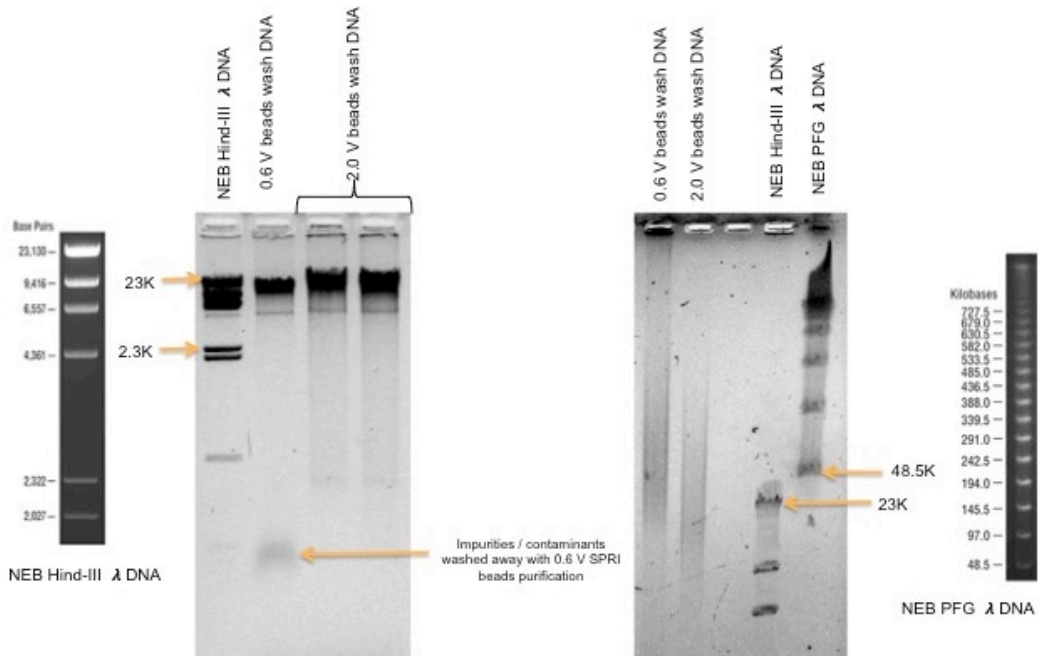

Fig. 4: A regular 1% TBE agarose gel (left) and a 1 % agarose gel run of pulsed-field (right) showing the 0.6V followed by 2 .0V SPRI beads extracted DNA. We loaded 10x more of the 0.6V than the 2.0V SPRI beads sample in order to visulise the DNA.

The Gel image shows the effect of low volume (0.6 V) SPRI beads purification of rust DNA to remove some contaminants invisible in the UV spectrum.

An optional 0.9 v SPRI beads size selection.

Step 35.

The 'low volume of purified DNA is pure enough for the sequencing', but to enrich high molecular weight DNA, I wash DNA with 0.9V homemade SPRI beads, the protocol and recipe for SPRI beads solution can be found here.

This help in removing smaller DNA fragments and enrich DNA molecules larger than 3-4 kb. The DNA recovery after 0.9V wash would 60-70 percent DNA, so make sure that you start with enough DNA so end up with enough size selected DNA for 1 D genomic library preparation.

Nanopore seqeuncing

Step 36.

I used this final DNA for 1 D genomic library (SQK-LSK108) preparation and sequencing on a FLO-MIN-106 (R9.4) with MinKNOW 1.17.23. I sequenced one wheat stripe rust (*Puccinia striiformis*) isolate and got 6.5 Gb data with a read N50 25K, which was one of the bast Nanpore run we got so for with rust DNA.