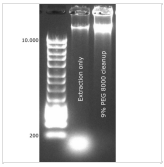

Jun 08, 2020

High-molecular weight DNA extraction and small fragment removal of *Ascochyta lentis*

Forked from High-molecular weight DNA extraction from challenging fungi using CTAB and gel purification

Johannes Wolfram JWD Debler[1], Ashley Jones[2], Ramawatar Nagar[2], Anna Sharp[2], Benjamin Schwessinger[2]

[1]Curtin University; [2]Australian National University

1 Works for me dx.doi.org/10.17504/protocols.io.bgw5jxg6

Johannes Wolfram Debler
Curtin University

ABSTRACT

Extracting pure high-molecular weight DNA from some fungal species is difficult due to the presence of polysaccharides and potentially other compounds which biochemically mimic DNA or interfere with the DNA extraction process. Such compounds can co-elute with DNA in many extraction methods, being difficult to separate fom the DNA. Although the contaminant may not be detected by spectrophotometers or fluorometric devices, it substantially interferes with long-read DNA sequencing, such as Oxford Nanopore Technologies. To partially resolve this, a protocol is presented with some updates to current strategies and incorporates a small fragment removal step using Polyethylene Glycol. Using this protocol, we have been successfully sequencing the lentil pathogen *Ascochyta lentis* with a MinION (Oxford Nanopore Technologies). Sequencing yields have surpassed 13 gigabases with an N50 of approximately 15 kb. To increase sequencing output, more work is needed to identify and remove the elusive contaminants.

DNA extraction modified from: https://www.protocols.io/view/high-molecular-weight-dna-extraction-from-challeng-5isg4ee

PEG small fragment elimination after: https://www.protocols.io/view/size-selective-precipitation-of-dna-using-peg-amp-7erhjd6

DOI

dx.doi.org/10.17504/protocols.io.bgw5jxg6

PROTOCOL CITATION

Johannes Wolfram JWD Debler, Ashley Jones, Ramawatar Nagar, Anna Sharp, Benjamin Schwessinger 2020. High-molecular weight DNA extraction and small fragment removal of Ascochyta lentis. **protocols.io** dx.doi.org/10.17504/protocols.io.bgw5jxg6

FORK FROM

Forked from High-molecular weight DNA extraction from challenging fungi using CTAB and gel purification, Ashley Jones

CREATED

May 28, 2020

LAST MODIFIED

Jun 16, 2020

PROTOCOL INTEGER ID

37565

GUIDELINES

This research builds on the work of Ramawater Nagar and Benjamin Schwessinger, as well as John Tyson; a warm thanks for their contributions.

This protocol is based on the following protocols below.

Nagar, R. and Schwessinger, B. (2018). Multi-step high purity high molecular weight DNA extraction protocol from challenging fungal tissues. *Protocols.io*

Arseneau et al. (2017). Modified low-salt CTAB extraction of high-quality DNA from contaminant-rich tissues. *Molecular Ecology Resources* **17** (4), 686-693.

Xin, Z. and Chen, J. (2012). A high throughput DNA extraction method with high yield and quality. *Plant Methods* **8**, 26.

https://www.longreadclub.org/mountain-protocol/

MATERIALS TEXT

Reagents
Cetrimonium bromide (CTAB)
Chloroform: isoamyl alcohol (24:1)
Ethylenediaminetetraacetic acid (EDTA)
Ethanol (70%)
Sodium chloride (NaCl)
Proteinase K (20 mg/mL)
Polyvinylpyrrolidone (PVP) 40,000
RNAse A (20 mg/mL)
Trisaminomethane hydrochloride (Tris-HCl) pH 8
Water, high-purity (e.g. Milli-Q system)
Water, nuclease-free
Polyethylene Glycol 8000
Yeast Extract
Glucose

Special Equipment
1.5 mL DNA LoBind Eppendorf tube (optional)
Centrifuge for 15 mL Falcon tubes (up to 5,000 rcf)
Mortar and pestle
Water bath

PREPARATION

1 Prepare about **500 mg of fungal mycelia** grown in liquid media (YEG, 0.5% Yeast Extract, 5% Glucose). Remove media by filtering through a sterile milk filter disk. Wash mycelia with Milli-Q water while on the filter and keep frozen in liquid nitrogen.

2 Set a water bath to 55°C. This will be used to dissolve 2% CTAB solutions and to preciptate DNA with CTAB.

3 Freshly prepare lysis and precipitation buffers.

Lysis buffer
- 5 mL per 500 mg of sample.

Citation: Johannes Wolfram JWD Debler, Ashley Jones, Ramawatar Nagar, Anna Sharp, Benjamin Schwessinger (06/08/2020). High-molecular weight DNA extraction and small fragment removal ofÂÂ Ascochyta lentis. https://dx.doi.org/10.17504/protocols.io.bgw5jxg6

Component	MW	Stock	Quantity (5 mL)
2% CTAB (w/v)	364.45	powder	0.10 g
100 mM Tris-HCl (pH 8)	157.60	1 M	0.50 mL
20 mM EDTA (pH 8)	292.24	0.5 M	0.20 mL
1.2 M NaCl	58.44	5 M	1.20 mL
1% PVP	40,000	10%	0.50 mL
Milli-Q water	-	-	2.60 mL

- *EDTA is a metal ion chelator. By binding to Mg^{2+}, DNase activity is stopped as it is dependent on Mg2+.*

Precipitation buffer

- 10 mL per 500 mg of sample.

Component	MW	Stock	Quantity (10 mL)
2% CTAB (w/v)	364.45	powder	0.20 g
100 mM Tris-HCl (pH 8)	157.60	1 M	1 mL
20 mM EDTA (pH 8)	292.24	0.5 M	0.40 mL
Milli-Q water	-	-	8.60 mL

The DNA extraction process appeared unaffected when the lysis and precipitation buffers were not adjusted to pH 8. Note Tris-HCl and EDTA stock solutions are already at pH 8.

9% PEG8000 small fragment elimination buffer

- ~300 uL per 500 mg of sample.

Component	MW	Stock	Quantity (10 mL)
9% PEG 8000	8000	18%	5 mL
1M NaCl	58.44	5 M	2 mL
10 mM Tris-Cl (pH 8.0)	157.60	1 M	100 uL
Milli-Q water	-	-	2.9 mL

4 Vortex lysis and precipitation buffers, then place both in the 55°C water bath to dissolve CTAB. Further vortexing or inverting may be needed to dissolve all CTAB. Leave buffers at 55°C until needed.

SAMPLE LYSIS

5 Place a sterile mortar and pestle into an insulated container. Pour liquid nitrogen into the container to chill the mortar and pestle.

Citation: Johannes Wolfram JWD Debler, Ashley Jones, Ramawatar Nagar, Anna Sharp, Benjamin Schwessinger (06/08/2020). High-molecular weight DNA extraction and small fragment removal ofÃÂ Ascochyta lentis. https://dx.doi.org/10.17504/protocols.io.bgw5jxg6

- *The lid of an ice box can be used. If liquid nitrogen is later poured directly onto the spores, spores disperse all over the pestle and potentially the table.*

6 Add mycelium to the mortar and grind for approximately 1-2 min. Keep the mortar and pestle chilled by having liquid nitrogen within the surrounding container.

7 Transfer the ground mycelium to **5 mL lysis buffer** in a 15 mL falcon tube.

8 Add RNAse A and Proteinase K to the solution:

Enzyme	Stock	Quantity (5 mL lysis buffer)
100 µg/mL RNAse A	10 mg/mL	50 µL
100 µg/mL Proteinase K	20 mg/mL	25 µL

- *RNAse A was added earlier than previous protocols and concentration doubled as RNA has been still present after extraction. Athough RNAse A concentration is generally reccomended to be 1-100 µg/mL, Qiagen DNeasy plant kits use as much as 2,000 µg/mL. RNAse T has less DNase activity (still present), but was less effective at degrading fungal RNA.*
- *RNAse A activity is not Mg^{2+} dependent and doesn't appear to require metal ions as cofactors. Similarily, Proteinase K is still active in EDTA, as the two binding sites for Ca^{2+} are not directly involved in the proteolysis catalytic mechanism.*
- *Proteinase K and RNAse A can co-exist in the same solution. RNAse A is highly resistant to proteolysis by Proteinase K. Both are recommended to be 50-100 µg/mL. RNAse A is active at temperatures15-70°C (optimal at 60°C), pH 6-10 (optimal pH 7.6). Proteinase K is active at temperatures 20-60°C (optimal 50-60°C), pH 4-12 (optimal pH 8).*

9 Vortex and invert the solution. Incubate at 55°C for at least 1 h.

CHLOROFORM CLEAN-UP

10 Add an **equal volume of chloroform: isoamyl alcohol (24:1, v/v)** and mix by inverting 10-15 times. Ensure the organic and aqueous phases become mixed at least temporarily.

11 Separate the phases by centrifuging at **5,000 rcf for 10 min at 20°C**. Transfer the upper aqueous phase to a new 15 mL Falcon tube.

Chloroform is denser than water, will mix with the oragnic phase which settles to the bottom and the

Citation: Johannes Wolfram JWD Debler, Ashley Jones, Ramawatar Nagar, Anna Sharp, Benjamin Schwessinger (06/08/2020). High-molecular weight DNA extraction and small fragment removal ofÂÂ Ascochyta lentis. https://dx.doi.org/10.17504/protocols.io.bgw5jxg6

aqueous phase is at the top. Some protocols recommend centrifuging at 12,000 rpm. Not tested, however if centrifugation hasn't been sufficient the interphase will be cloud-like and poorly compacted, with oraganic substances still in the aqueous phase.

12 Repeat the chloroform: isoamyl alcohol clean (equal volume).

DNA PRECIPITATION

13 Add 2
volumes of precipitation buffer,
mix by inverting. Incubate at
55°C for 1 h
or until white crystals of CTAB-DNA complex can be observed floating inside the tubes.

14 Centrifuge at **5,000 rcf for 30 minutes at 20°C** to pellet crystals.

15 Carefully decant the supernatant as soon as possible, without disturbing the pellet. Care must be taken as the pellet is fragile, being easily dislodged.

16 Add enough freshly prepared **70% ethanol** to cover the pellet (**~5 mL**). Let the pellet soak for 15 min at room temperature to dissolve excess salts and CTAB.

17 Centrifuge at **5,000 rcf for 10 min**. Carefully decant the supernatant as soon as possible, without disturbing the pellet.

18 Air-dry the pellet for 10-15 min, or until all ethanol has evaporated.

19 Dissolve DNA with a maximum of **300 µL in 0.1x TE** in nuclease-free water. Avoid pipette mixing as much as possible to prevent DNA shearing. Gentle flicking of the tube and incubating at room temperature is ideal. Wide-bore pipette tips are also an option.

- *Volume is based on loading the sample across a whole PippinHT (Sage Science) casette at later steps.*
- *If planning to deviate from this protocol and perform other clean-ups or enzymatic digestions, it is recommended to dissolve the pellet in 50-500 µL of 10 mM Tris (pH 8.0). This keeps the DNA buffered and in solution.*
- *This step can take a couple of days!*

20 Transfer DNA to a 1.5 mL DNA LoBind Eppendorf tube.

Citation: Johannes Wolfram JWD Debler, Ashley Jones, Ramawatar Nagar, Anna Sharp, Benjamin Schwessinger (06/08/2020). High-molecular weight DNA extraction and small fragment removal ofÃÂ Ascochyta lentis. https://dx.doi.org/10.17504/protocols.io.bgw5jxg6

21 Quantify the DNA on a Nanodrop and a Qubit fluorometer (dsDNA broad-range assay) (both instruments from Thermo Fisher Scientific). The DNA is still crude and will likely have RNA and other contaminants present.

For Nanodrop, use 1 µL. However, Qubit fluorometer accuracy is highly dependent on the accuracy of the amount pipetted. Use 2 µL when sample is plentiful. Using 1 µL is prone to pipetting errors.

22 Store DNA at 4°C to prevent cycles of freeze-thawing that shear the DNA.

No effects on DNA integrity have been noticed for samples stored at 4°C for extended periods.

PEG small fragment elimination

23 Combine **250 uL DNA** with **250 uL 9% PEG buffer** and incubate for 30 min at room temperature.

24 Centrifuge for **30 min at 10,000 rcf at room temperature.**

25 Carefully remove the supernatant without disturbing the pellet.

26 Wash pellet by covering it with **300-500 uL 80% Ethanol**, incubate for **5 minutes at room temperature** then spin for **5 minutes at 10,000 rcf at room temperature**. Repeat this step one more time.

27 Remove the ethanol and allow the pellet to air dry for a couple of minutes. Resuspend pellet in **250 uL 0.1x TE**.

- *It can take a couple of days for the pellet to fully rehydrate.*

28 Quantify the DNA on a Nanodrop and a Qubit fluorometer (dsDNA broad-range assay) (both instruments from Thermo Fisher Scientific). The DNA should be pure, free of contaminants.

- *For Nanodrop, use 1 µL. Qubit fluorometer is highly dependent on the accuracy of the amount pipetted. Use 2 µL when sample is plentiful. Using 1 µL is prone to pipetting errors.*
- *For pure DNA, Nanodrop: Qubit quantifications are 1:1.*
- *Expect 10-30% recovery relative to total input.*

Citation: Johannes Wolfram JWD Debler, Ashley Jones, Ramawatar Nagar, Anna Sharp, Benjamin Schwessinger (06/08/2020). High-molecular weight DNA extraction and small fragment removal ofÃÂ Ascochyta lentis. https://dx.doi.org/10.17504/protocols.io.bgw5jxg6

29 Store DNA at 4°C to prevent cycles of freeze-thawing that shear the DNA.

No effects on DNA integrity have been noticed for samples stored at 4°C for extended periods.

Citation: Johannes Wolfram JWD Debler, Ashley Jones, Ramawatar Nagar, Anna Sharp, Benjamin Schwessinger (06/08/2020). High-molecular weight DNA extraction and small fragment removal ofÃÂ Ascochyta lentis. https://dx.doi.org/10.17504/protocols.io.bgw5jxg6