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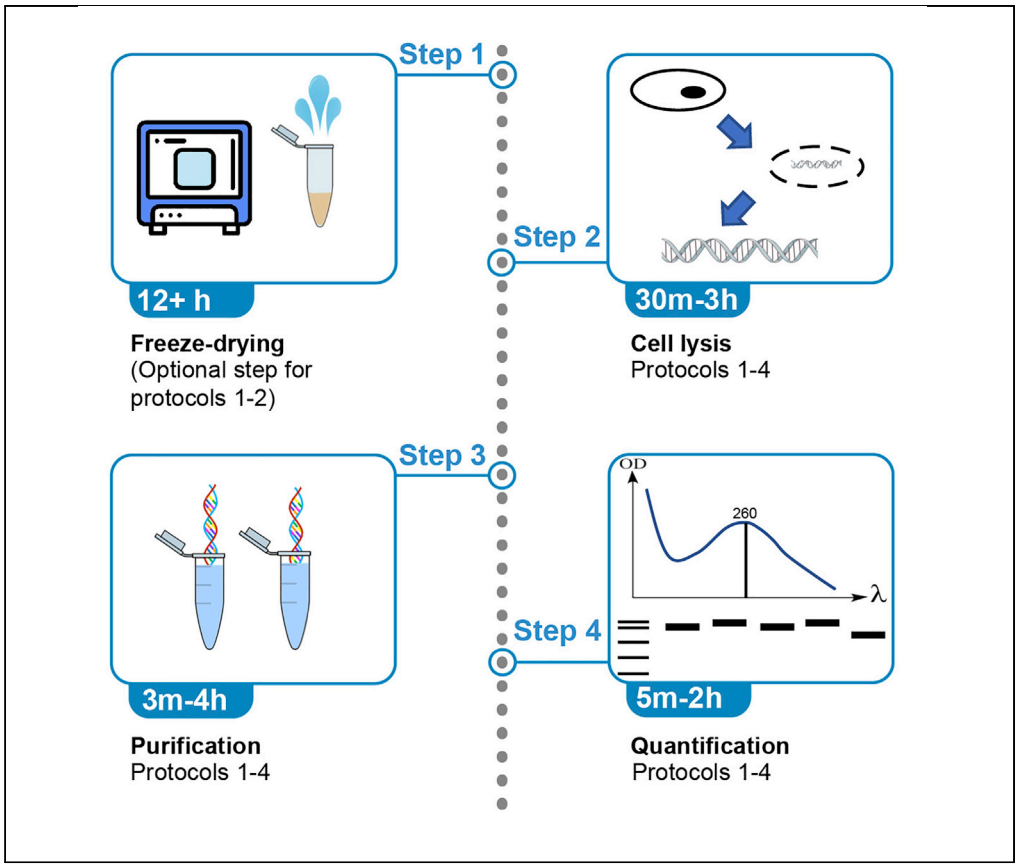
Orthogonal protocols for DNA extraction from filamentous fungi



There are few protocols available for DNA extraction from fungi. Here we present four complementary protocols for extraction of genomic DNA from fungi. We quantify the efficacy of extractions and compare eight species from five filamentous fungal genera, including both basidiomycetes and ascomycetes. These protocols should be useful for extraction of DNA from a variety of filamentous fungi.

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Highlights

Comparison of four DNA extraction protocols for fungi

Freeze-drying greatly increases DNA yield

CTAB extraction is optimal for high yield and fragment length

Chelex is a quick technique for DNA extraction from fungi

Conlon et al., STAR Protocols 3, 101126
March 18, 2022
https://doi.org/10.1016/j.xpro.2022.101126

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Orthogonal protocols for DNA extraction from filamentous fungi

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https://doi.org/10.1016/j.xpro.2022.101126

SUMMARY

There are few protocols available for DNA extraction from fungi. Here we present four complementary protocols for extraction of genomic DNA from fungi. We quantify the efficacy of extractions and compare eight species from five filamentous fungal genera, including both basidiomycetes and ascomycetes. These protocols should be useful for extraction of DNA from a variety of filamentous fungi.

For complete details on the use and execution of this protocol, please refer to Conlon et al. (2021).

BEFORE YOU BEGIN

The protocols below describe 4 different DNA extractions performed for fungi. Our extractions used fungi grown *in vitro* on solid media, but extractions from liquid media should also be possible. For Protocols 1 and 2 there was an optional freeze-drying step. Protocol 3 is based on Walsh et al. (1991) while Protocol 4 is based on Suenaga and Nakamura (2005). In addition to comparing effectiveness between protocols, we also quantify the benefit of freeze-drying for DNA yield. All necessary reagents should be prepared in advance. For the CTAB protocol, isopropanol should be stored at –20°C.

The protocols in order are:

Protocol 1: CTAB (hexadecyltrimethylammonium bromide)

Protocol 2: Qiagen (Germany) DNeasy plant pro mini kit

Protocol 3: Chelex

Protocol 4: Chelex with proteinase K

Harvesting of fungal tissue

Timing: 5 min

1. Weigh an empty 1.5 mL microcentrifuge tube.
   a. Record weight to allow calculation of starting weight.

2. Using a sterile scalpel, scrape ~50 mg (CTAB and Qiagen) or ~10 mg of mycelium from agar.
   a. In our extractions, starting weights varied from 30–75 mg (CTAB and Qiagen) and 5–11 mg (Chelex).
3. Place mycelium into the tube and weigh the full tube.
   a. Subtract empty weight from full weight to calculate starting material.
4. Store sample at –20°C.

Freeze-drying of fungal samples

Timing: 12+ h

The freeze-drying process is the removal of ice or other frozen solvents from a material through the process of sublimation and consists of three stages: freezing, vacuum, and then drying. This step reduces DNA fragmentation and increases the quantity of DNA.

5. Completely freeze the tissue.
   a. This is achieved either by using liquid nitrogen or by storing in at -20 for 2–3 h.
6. Tubes are sealed with parafilm and 3–5 small holes are made in the lid using a needle.
   a. Parafilm ensures that the caps do not snap open during the freeze-drying process.
7. Store samples at –20°C.
8. Run the samples through the freeze-drying process using a freeze-dryer.
   a. For our samples, the vacuum was set to 0.018 mbar and temperature to −58°C.
9. Samples can be stored in a sealed container at 18°C–27°C for up to 1 year.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Leucoagaricus gongylophorus	*Acromyrmex echinatior* ant colony (Panama)	Ae420B (420)
Leucoagaricus gongylophorus	*Acromyrmex echinatior* ant colony (Panama)	Ae360 (360)
Termitomyces sp.	*Macrotermes bellicosus* termite colony (Ivory Coast)	IC0027 (27)
Termitomyces sp.	*Macrotermes bellicosus* termite colony (Ivory Coast)	IC0010 (10)
Podaxis carcinomalis	*Trinervitermes* sp. mound (Australia)	AQ 795752 (P)
Pseudoxylaria sp.	*Macrotermes natalensis* termite colony (South Africa)	X802 (X)
Pseudoxylaria sp.	*Odontotermes* sp. termite colony (Ivory Coast)	IC0057 (X57)
Escovopsis sp.	*Paratrachymyrmex* ant colony (Panama)	*Escovopsis* 176705 (E)
Chemicals, peptides, and recombinant proteins		
Chelex 100	Sigma-Aldrich, USA	Cat# C7901
Proteinase K	QIAGEN, DE	Cat# 19131
CTAB	Sigma-Aldrich, USA	Cat# H6269
Tris	Merck, USA	Cat# GE17-1321-01
NaCl	VWR, USA	Cat# 27810.295
EDTA	Sigma-Aldrich, USA	Cat# 03685
RNase A	QIAGEN, DE	Cat# 195939
β-Mercaptoethanol.	Sigma-Aldrich, USA	Cat# STBD6281V
Chloroform	Merck, USA	Cat# 1.02445
Isoamylalcohol	Merck, USA	Cat# 1.00979
Phenol:Chloroform:Isoamyl alcohol	Fisher Scientific, USA	Cat# BP1752I

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Agarose	Th. Geyer, Germany	Cat# 9953.0500
TAE Buffer	VWR, USA	Cat# K915
1kb DNA Extension Ladder	Fisher Scientific, USA	Cat# 11568626
DNA gel loading dye	Thermo Fisher Scientific, USA	Cat# R0611
GelRed nucleic acid stain	Merck, USA	Cat# SCT123
96% Ethanol	VWR, USA	Cat# 83804.360
EB Buffer	QIAGEN, DE	Cat# 19086
Critical commercial assays		
Qiagen DNeasy PlantPro Mini Kit	QIAGEN, DE	Cat# 69204
Qubit dsDNA BR Assay Kit	Thermo Fisher Scientific, USA	Cat# Q3265G
Other		
Qubit 4 Fluorometer	Thermo Fisher Scientific, USA	Cat# Q33238
Nanodrop 1000 Spectrophotometer	Thermo Fisher Scientific, USA	Cat# ND-1000
Christ Alpha 1–4 LDplus (freeze-dryer)	VWR, USA	n/a
Data		
QC data for DNA extractions	Mendeley Data	https://doi.org/10.17632/683ybmk665.1

MATERIALS AND EQUIPMENT

CTAB buffer		
Reagent	Final concentration	Amount
CTAB	2%	20 g
NaCl	1.4 M	82 g
Tris	0.1 M	12.1 g
EDTA	20 mM	5.8
ddH_2O	n/a	Bring volume to 1 L
Total	**n/a**	**1 L**
Adjust pH to 8 using HCl or NaOH and store at 18°C–27°C in a ventilated cupboard for up to one year.		

⚠ CRITICAL: CTAB and EDTA should be handled with protective gloves in a fume hood.

Chloroform: Isoamyl alcohol		
Reagent	Final concentration	Amount
Chloroform	24	48 mL
Isoamyl alcohol	1	2 mL
Total	**24:1**	**50 mL**
Store at 18°C–27°C in a ventilated cupboard for up to one year.		

⚠ CRITICAL: Chloroform and isoamyl alcohol should be handled with protective gloves in a fume hood.

5M NaCl		
Reagent	Final concentration	Amount
NaCl	5M	292.2 g
dd H_2O	n/a	Bring volume to 1 L
Total	**5M**	**1 L**
Store at 18°C–27°C for up to one year.		

5% Chelex solution		
Reagent	Final concentration	Amount
Chelex 100	5%	5 g
ddH_2O	n/a	Bring volume to 1 L
Total	**5%**	**1 L**

Store at 4°C with a magnetic stirrer autoclaved in a bottle for up to one year.

0.8% Agarose solution		
Reagent	Final concentration	Amount
Agarose	0.8%	8 g
TAE Buffer	n/a	Bring volume to 1 L
Total	**0.8%**	**1 L**

Make fresh daily and keep at 60°C until pouring.

Loading buffer		
Reagent	Final concentration	Amount
GelRed	0.5%	5 μL
Diluted loading buffer	n/a	1 mL
Total	**0.8%**	**1.005 mL**

Keep at 18°C–27°C and protected from light for up to one month.

STEP-BY-STEP METHOD DETAILS

Protocol 1: CTAB extraction for fungi

Timing: 7 h

The CTAB DNA extraction method is cheap, effective and applicable for a wide range of applications including DNA barcoding, shotgun sequencing and long-read sequencing. The protocol utilizes organic solvents and the harmful nature of some of the solvents, combined with the relatively long time to complete the protocol, can therefore be a limitation of using this method.

1. Harvest fungal material as outlined in Before you begin Steps 1-4.
2. Homogenize tissue
   a. Add 200 μL CTAB buffer to sample and grind tissue with a micropestle.
      i 1.5 mL Eppendorf tubes often work best
   b. Add 200 μL CTAB buffer to sample and grind tissue with a pipette tip with the end melted
      i Place end of a 1000 μL pipette tip into the flame of an ethanol or Bunsen burner until the hole is sealed
      ii Allow the tip to cool. The plastic end often becomes cloudy as it cools
   c. Homogenize using a Qiagen TissueLyser (or other lysis machine)
      i Add glass beads or a heavy metal bead to the tube (2 mL Eppendorf tubes often work best for the heavy beads)
      ii Run TissueLyser at 24 Hz for four min
3. Add 500 μL CTAB buffer, 7 μL Proteinase K, 7 μL RNase A and 7 μL β-Mercaptoethanol.
4. Incubate on a rotor at 65°C for at least 3 h (1 h if samples were freeze-dried).
5. Centrifuge for 5 min at 20,000×*g*.
6. Transfer supernatant to a new tube.

7. Add 700 μL phenol-chloroform-isoamyl alcohol (25:24:1).
8. Mix by turning upside down 100 times and incubate for 5 min at 18°C–27°C.
   a. Vortexing for 5 s is also possible but increases the risk of shearing DNA
9. Centrifuge for 30 min at 20,000×*g* and 20°C.
10. Transfer the upper phase, avoiding the white interphase, to a new tube.
11. Add 700 μL chloroform:isoamyl alcohol (24:1).
12. Mix by turning upside down 100 times and incubate for 5 min at 18°C–27°C.
    a. Vortexing for 5 s is also possible but increases the risk of shearing DNA
13. Centrifuge for 15 min at 20,000×*g* and 20°C.
14. Transfer upper phase, avoiding the white interphase, to a new tube while measuring supernatant volume.
15. Add 1/3 supernatant volume of 5M NaCl and 2/3 supernatant volume of ice-cold isopropanol to the tube.
    a. This step brings the DNA out of solution. While the isopropanol does not have to be ice-cold, it helps to improve overall yield.
16. Mix by turning upside down 20 times and centrifuge for 30 min at 20,000×*g* and 20°C.
17. Discard supernatants.
    a. Avoid disturbing any pellet with a pipette tip.
18. Add 750 μL 70% Ethanol and centrifuge for 3 min at 20,000×*g*.
    a. The use of ice-cold ethanol can improve yield.
    b. Can be repeated to increase purity.
19. Discard supernatants.
    a. Avoid disturbing any pellet with a pipette tip.
20. Air-dry pellets then add 50–100 μL EB buffer (or other elution buffer).
    a. Maximum time for air-drying is 10 min. Be sure to properly remove ethanol with a 10 μL pipette and the remaining ethanol will evaporate within a few minutes.
    b. Allow the sample to dissolve for at least 1 h at 18°C–27°C or for 12 h at 4°C.

Note: Unless otherwise indicated, centrifugation steps are at 18°C–27°C.

Protocol 2: Qiagen DNeasy plant pro mini kit

Timing: 1 h 20 min

DNA extraction using the Qiagen (Germany) DNeasy plant pro mini kit. While more expensive than the other protocols, this kit is simple and relatively quick to use and is also available in a 96-well format.

21. Using starting material harvested according to Before you begin Steps 1-4, we followed the manufacturer's instructions with the following options:
22. All centrifuge steps at 20°C.
23. Lysis using a Qiagen TissueLyser LT: 4 min at 24 Hz.
24. Step 5: addition of 250 μL Buffer CD2.

Protocol 3: Chelex

Timing: 25 min

The Chelex extraction is cheap and very quick to extract from a lot of samples. The Chelex resin inhibits DNases which are not denatured by boiling. This protocol works very well for DNA barcoding and could potentially be used for shotgun sequencing. Extractions can be performed in a 96 well plate meaning it can be possible to extract DNA from 96 samples in under 1 h.

25. Place 5% Chelex solution on magnetic stirrer.
26. Following Before you begin Steps 1-4, harvest fungal mycelium into a 250 μL PCR tube.
27. Add 200 μL of 5% Chelex.
28. Vortex tubes for 15 s.
29. Incubate in a PCR machine at 99.9°C for 15 min.
30. Centrifuge tubes at 3,300×*g* for 3 min.
31. Transfer 100 μL of supernatant to a new tube.

Protocol 4: Chelex with proteinase K

Timing: 40 min

This method is like the above Chelex protocol but with the addition of Proteinase K to assist lysis of the fungal cells and increase DNA yield.

32. Place 5% Chelex solution on magnetic stirrer.
33. Following Before you begin Steps 1-4, harvest 10–20 mg of fungal mycelium into a 250 μL PCR tube.
34. Add 200 μL of 5% Chelex with 20 mg/mL of Proteinase K.
35. Vortex tubes for 15 s.
36. Incubate in a PCR machine at 65°C for 30 min.
37. Centrifuge tubes at 3,300×*g* for 3 min.
38. Transfer 100 μL of supernatant to a new tube.

Agarose gel electrophoresis

Timing: 120 min

We used agarose gel electrophoresis to provide a qualitative assessment of the length of DNA fragments in our extracts.

39. Pour 0.7%–1% (0.8 % was used in this experiment) into a gel tray.
40. Mix 0.5 μL of 1 kb DNA Extension Ladder, 2 μL loading buffer and 2.5 μL H2O then add to the first well.
41. Mix 3 μL loading buffer with 2 μL sample DNA and add to individual wells.
42. Run gel at 60–70 V for approximately 90 min.

EXPECTED OUTCOMES

Based on our results, the protocols are expected to result in 30–60 ng DNA per mg of starting material for the CTAB and Chelex protocols and 6–8 ng DNA per mg starting material for DNeasy (Table 1). While freeze-drying did not have a significant effect on DNA yield (Figure 1A) or purity (Figures 1B and 1C), it did result in longer fragments, which would be suitable for long-read sequencing

Table 1. Mean (± Standard Error) extraction time, yield and integrity across for each method

Preparation	Extraction method	Time (h)	DNA yield per starting weight ng/mg	260/280	260/230
None	Chelex	0.3	36.097 (8.454)	2.064 (0.325)	1.911 (1.213)
None	Chelex with ProteinaseK	0.6	35.340 (7.359)	1.439 (0.074)	0.549 (0.045)
None	CTAB	7	61.041 (18.885)	1.979 (0.056)	0.999 (0.174)
None	DNeasy	1.25	7.566 (9.336)	2.120 (0.255)	0.586 (0.117)
Freeze-drying	CTAB	4	39.801 (16.996)	2.019 (0.066)	1.105 (0.181)
Freeze-drying	DNeasy	1.25	6.603 (2.861)	1.711 (0.255)	1.404 (0.540)

Individual results available from Mendeley Data

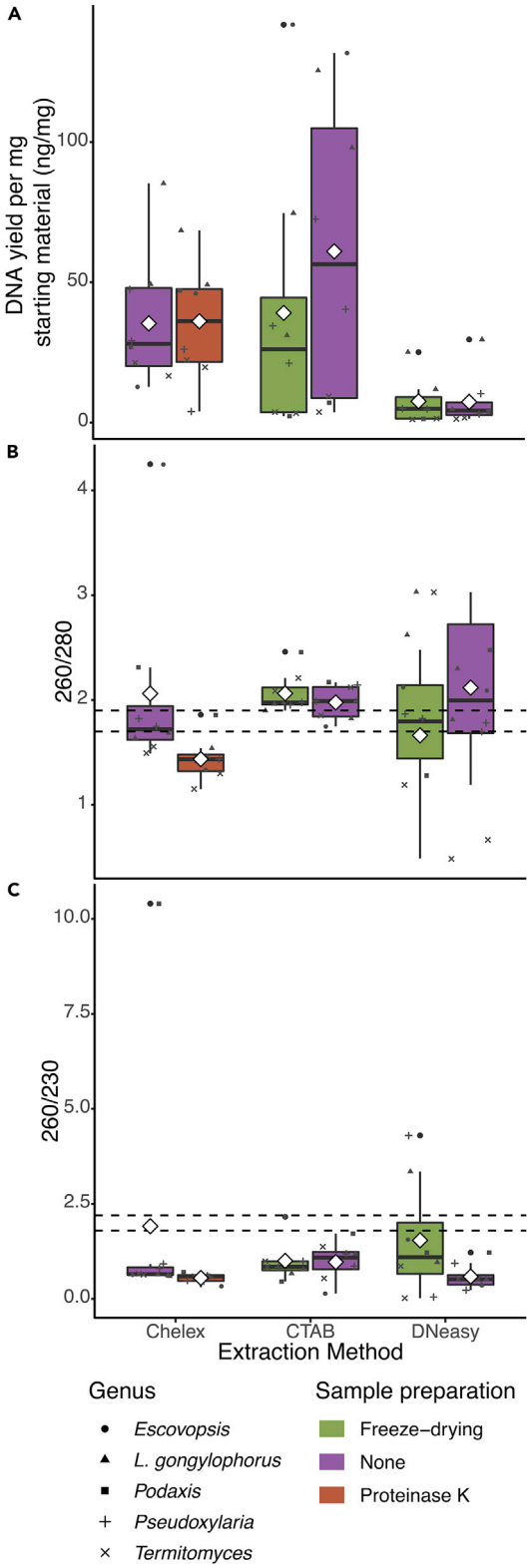

Figure 1. Quantification of DNA extracts using Qubit and Nanodrop

(A) DNA yield per mg of starting material was highest for CTAB and Chelex extractions. However, both methods exhibited much more variation in yield than the DNeasy kit. White diamonds indicate mean values, thick black lines indicate median values, boxes correspond to the 1st and 3rd quartiles while whiskers correspond to the lowest value or 1.5× the Interquartile Range (IQR), whichever is smallest.

(B) Protein and RNA contamination (quantified using 260/280 ratio) ratios were relatively consistent for all protocols. However, the readings for the DNeasy kit were much more variable than the CTAB and Chelex extractions. The addition of Proteinase K to the Chelex extraction appeared to increase the protein contamination. Dashed lines show the boundaries for an optimal value (1.7 and 1.9). White diamonds indicate mean values, thick black lines indicate median values, boxes correspond to the 1st and 3rd quartiles while whiskers correspond to the lowest value or 1.5× the Interquartile Range (IQR), whichever is smallest.

(C) All extracts appeared to contain some reagent contamination (quantified using 260/230 ratio). While the results were consistent for most methods, the DNeasy protocol with optional freeze-drying exhibited much higher variation. Dashed lines show the boundaries for optimal values (1.8 and 2.2). White diamonds indicate mean values, thick black lines indicate median values, boxes correspond to the 1st and 3rd quartiles while whiskers correspond to the lowest value or 1.5× the Interquartile Range (IQR), whichever is smallest.

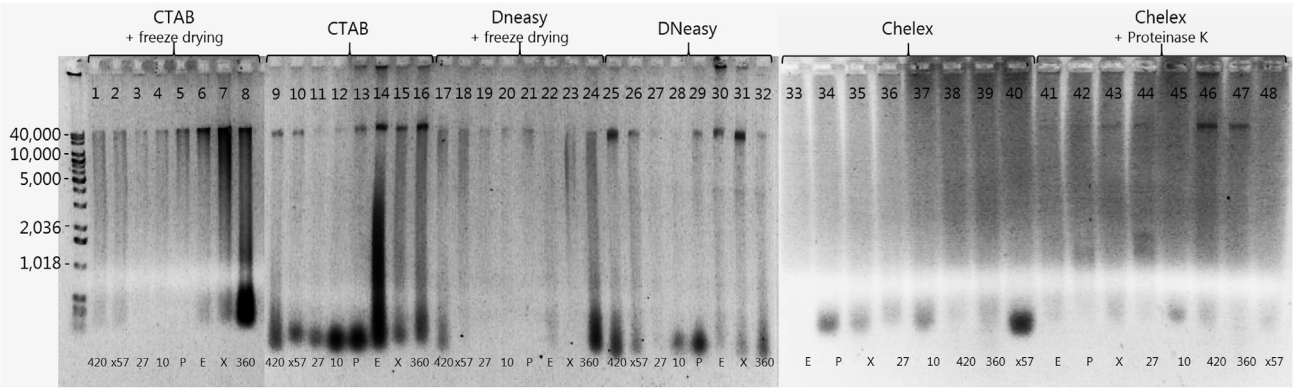

Figure 2. Qualitative assessment of the genomic DNA demonstrated that the CTAB protocol was optimal for the extraction of high-molecular weight DNA across fungal species

Freeze-drying was found to further improve the efficiency of recovery by decreasing the intensity of the smears (degraded DNA). The Chelex extraction with the addition of Proteinase K also resulted in the recovery of relatively large fragments of DNA. As the elution volume used in each extraction affected the DNA concentration, Chelex extracts were run separately from the CTAB and DNeasy extracts. Lanes 1–16 represent the CTAB extraction protocol (1–8 with an initial freeze-drying step). Lanes 17–32 represent the DNeasy protocol (17–24 with an initial freeze-drying step). Lanes 33–40 represent the Chelex extraction. Lanes 40–48 represent the Chelex extraction with proteinase K added. Strain IDs are given in the key resources table. Image is a composite of two gels: one combining CTAB and DNeasy extractions and a second for Chelex extractions.

(Figure 2). The addition of Proteinase K to the Chelex extraction increased yield slightly (Figure 1A) and fragment length, meaning that this is potentially a good option for high-throughput DNA extraction for long-read sequencing. In order to represent the potential variability in yield and quality, as well as ease of replication, for each protocol, we performed each extraction once per strain.

QUANTIFICATION AND STATISTICAL ANALYSIS

DNA yield (ng/μL) was quantified using a Qubit 4 Fluorometer with a Broad Range dsDNA assay kit (Thermo-Fisher, USA). Protein and RNA contamination was assessed using a Nanodrop 1000 (Thermo-Fisher, USA) spectrophotometer by comparing the absorption ratio for λ260: λ280 (260/280) while reagent contamination was also assessed using the Nanodrop by comparing the absorption ratio for λ260: λ230 (260/230). DNA integrity and fragment size was assessed qualitatively using agarose gel electrophoresis (Table 1).

LIMITATIONS

The Chelex and DNeasy protocols are quick and result in enough DNA at a reasonable-enough purity for PCR or next-generation sequencing. However, the DNA fragment length was low for both protocols. The CTAB protocol results would therefore be preferable for long read sequencing and when DNA is extracted from limited amounts of starting material. CTAB has the added benefit of being more customizable than a kit. The Chelex protocol with the addition of Proteinase K appears to have produced relatively high concentrations of long-fragment DNA. This suggests that an added protein purification step could result in a quick, low-cost, and high-throughput method to extract DNA for long-read sequencing. We have, however, not attempted this here.

TROUBLESHOOTING

Problem 1

Incomplete phase separation in Steps 10 and 14 of CTAB extraction.

Potential solution

This can occur if the samples are not properly mixed before centrifugation, or if the samples are physically disturbed after centrifugation. In both cases, the solution is to mix and centrifuge the samples again.

For Step 14, this could also be caused by the transfer of the incorrect phase from Step 10. It can therefore be advisable to retain the remaining liquid from Step 10 until after Step 14. The solution here would be to return to Step 9, otherwise it is necessary to restart the protocol with fresh material.

Problem 2
High protein content in CTAB extract.

Potential solution
This is often due to transferring some of the white interphase with the supernatant in Steps 10 and 14. It is therefore best to pipette slowly and to not to take all of the supernatant when performing these steps.

Problem 3
Low DNA yield.

Potential solution
If the yield is low but the quality of the sample is good, it would suggest that the starting sample amount could be insufficient or that cell lysis was unsuccessful. Cell lysis can be checked by placing the sample under a microscope. Increase the amount of starting material and repeat the extraction, change lysis method, Step 2 for CTAB protocol, or add a freeze-drying step to ensure enough DNA for downstream application.

Problem 4
260/280 ratio is outside optimal boundaries of 1.7 and 1.9. A value above 1.9 could indicate the presence of RNA in the extract, while a value below 1.7 could indicate the presence of protein or other organic tissues in the sample.

Potential solution
Process the starting material according to the recommended protocol instructions to ensure thorough removal of proteins. An important step is to not use too much starting material and to be sure not to transfer any of the white interphase in the CTAB protocol (see problem 2). To remove protein contaminants, it might be necessary to add protease or perform an additional phenol extraction step. In addition, ensure that wash steps are performed carefully to eliminate carryover of contaminants into the final sample.

Problem 5
260/230 ratio is outside optimal boundaries of 1.8 and 2.2. A value below 1.8 could indicate contamination with reagents.

Potential solution
Follow the recommendations for processing different sample types. Process the starting material according to the recommended protocol instructions. For the CTAB extraction, phenol or chloroform contamination can occur if the interphase is disturbed during Step 10 or 14. While a low 260/230 is not ideal, it is often possible to still use extracts in downstream applications; this was the case for several of the samples used for shotgun sequencing in Conlon et al. (2021).

Problem 6
Your downstream application is not working.

Potential solution
The quality of your genomic DNA is poor, potentially degraded, or there might be contaminants in your extract. Be sure to not start with too much starting material and ensure sufficient washing steps in your protocol (Step 18 in CTAB protocol). There might be residual ethanol remains in the sample

when using ethanolic wash buffers for the final step in your DNA extraction. Be sure to thoroughly air dry the sample prior to adding the elution buffer. Use a 10 μL pipette to remove as much ethanol as possible before air drying.

Problem 7

Your DNA will not amplify in a PCR.

Potential solution

This can be due to the presence of PCR inhibitors in your extract. The addition of 0.004% Bovine Serum Albumin (BSA) to the PCR mix (0.1 μL for a 25 μL PCR) can help solve this.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Benjamin H. Conlon (benjamin.conlon@bio.ku.dk).

Materials availability

There are no newly-generated materials associated with this protocol.

Data and code availability

Original data are available from Mendeley Data: https://doi.org/10.17632/683ybmk665.1

ACKNOWLEDGMENTS

We would like to thank Sylvia Mathiasen for her assistance in the lab. This research was funded by a European Research Council Starting Grant (ELEVATE: ERC-2017-StG-757810) to J.Z.S. and the European Research Council Consolidator Grant (DEFEAT: ERC-CoG-771349) to M.P. The Ministerio de Ambiente, Republica de Panama provided permits for field research (SE/A-24-19) and sample exportation (SEX/A-41-19).

AUTHOR CONTRIBUTIONS

Study designed by B.H.C. and S.S. with inputs from M.P. and J.Z.S. Experimental work carried out by B.H.C. and S.S. This first draft of the manuscript was written by B.H.C. and S.S. All authors contributed to the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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