Version 2

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CTAB chloroform DNA extraction from ethanol-preserved filters V.2

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ABSTRACT

This protocol extracts high molecular weight genomic DNA from filters used to sample microbes from aquatic environments. It was developed for polycarbonate filters stored at ambient temperature in 100% ethanol, but works well on a wide variety of difficult environmental samples, including soils and sediments, as well as on animal, plant and insect tissues. If an alternative sample preservation method was used that also protects RNA (e.g. flash-freezing in liquid nitrogen followed by storage at -80degC), then this protocol can co-extract RNA.

In tests, this protocol resulted in higher yields and better integrity of genomic DNA compared with commercial extraction kits, at a fraction of the cost. The DNA extract obtained at the end is usually suitable for direct use in PCR, but for sensitive downstream applications (e.g. direct whole genome/metagenome sequencing) and for especially tricky sample types that contain high levels of potential inhibitors (e.g. humics, metals), an additional clean-up step with a commercial kit is recommended.

The protocol was based on a precipitation and re-suspension method adapted from Bramwell *et al.* (1995), with some of the modifications recommended by Lever *et al.* (2015). When citing, please also include these publications.

Bramwell, P.A., Barallon, R.V., Rogers, H.J., and Bailey, M.J. (1995) Extraction and PCR amplification of DNA from the rhizoplane, in Akkermans, A.D.L., Elsas, J.D.v. and Bruijn, F.J.d (eds.), *Molecular Microbial Ecology Manual*, Dordrecht: Kluwer Academic Publishers.

Lever, M.A, Torti, A., Eickenbusch, P., Michaud, A.B., Santl-Temkiv, T. and Jorgensen, B.B. (2015) A modular method for the extraction of DNA and RNA, and the separation of DNA pools from diverse environmental sample types. *Frontiers in Microbiology* 6: 476.

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WHAT'S NEW

Minor edits and clarification, plus recommendations for checking DNA quantity, integrity and purity.

KEYWORDS

DNA extraction, water, CTAB, high molecular weight DNA, ethanol preserved samples

IMAGE ATTRIBUTION

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MATERIALS TEXT

Equipment required:

1. Freezers for sample storage (-80°C and -20°C)
2. Freeze dryer to remove ethanol from samples (see safety warnings)
3. *[optional]* Benchtop homogenizer (bead beater) that can hold 2 mL screw-cap tubes, e.g. FastPrep-24 (MP Biomedicals), Precellys24 (Bertin Instruments), PowerLyzer 24 (MO BIO Laboratories), or TissueLyser II (Qiagen)
4. Oven with shaker or rotating rack for 55°C incubation (if not available, a heat block that holds 2 mL tubes or a water bath can be used instead)
5. Water bath for 37°C and 65°C incubations (can use the same water bath for both)
6. Benchtop chilled microcentrifuge (4°C)
7. Fumehood and chemical waste disposal arrangements for working safely with chloroform

Reagents required:

1. Lysis buffer: 30 mM Tris, 30 mM EDTA, pH 8 (3 mL of 1 M Tris pH 8, 6 mL of 0.5 M EDTA pH 8, up to 95 mL with nanopure water, which leaves 5 mL spare volume for subsequent addition of SDS)
2. [optional] Bulk beads for bead beating, e.g. Lysing Matrix A Bulk (Garnet) from MP Biomedicals (cat. no. 116540423, www.mpbio.com) or 0.7 mm Garnet Beads Bulk from Omni International (cat. no. 19-644, www.omni-inc.com)
3. 10% SDS (w/v)
4. Proteinase K stock solution: 20 mg/mL
5. Lysozyme stock solution: 1000 U/uL Ready-Lyse Lysozyme (epicentre)
6. 5 M NaCl
7. 10% CTAB (w/v) solution
8. Chloroform:isoamyl alcohol (24:1), molecular-grade
9. Isopropanol, molecular-grade
10. LPA, linear polyacrylamide solution (e.g. GenElute LPA from Sigma, cat. no. 5-6575)
11. 70% ethanol, freshly made with molecular-grade water
12. Molecular-grade water or molecular-grade TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), for resuspension. **TE buffer helps prevent DNA degradation so is preferable for long-term storage. However, some downstream applications may be inhibited by TE, in which case 10 mM Tris pH 8.5 (i.e. Qiagen buffer EB) or pure water can be used.*

SAFETY WARNINGS

The freeze-dryer used to remove ethanol must be suitable for flammable solvents, with a spark-proof pump and venting to an appropriate outlet (e.g. chemical fumehood). Standard freeze-dryers used purely for aqueous solutions are not appropriate due to the risk of explosion and fire.

This protocol includes the use of chloroform. All steps involving chloroform must be performed in an appropriate chemical fumehood, and chemical waste disposal arrangements must be in place to handle chlorinated solvents.

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Preparation

1 Samples collected in the field should be preserved in 100% molecular-grade ethanol, in 2 mL screw-cap cryotubes. Upon arrival at the laboratory, they should be stored at **-20 °C** .

This protocol was originally developed for polycarbonate filters, 47 mm diameter, 0.2 µm or 0.4 µm pore size, through which 50-200 mL pond water had been pushed to collect suspended microorganisms. Whole filters were folded with sterile forceps and placed in 2 mL tubes of 100% ethanol.

The protocol has since been used on sediments, soils, bird faeces, and various vertebrate and invertebrate tissues. For these sample types, 25-50 mg were generally sufficient, and where inhibitors were a concern, greater success was obtained with smaller starting amounts (5-10 mg).

2 Prior to DNA extraction, ethanol should be removed from the tubes using a freeze dryer with an operating temperature below **-80 °C** (exact protocol will depend on the model of freeze dryer). Tubes should be kept cold at all times to prevent DNA degradation. After freeze drying, tubes should be kept at **-80 °C** until processing. Storage time of dry tubes should be kept to a minimum. To protect samples from degradation, lysis buffer can be added immediately after samples are removed from the freeze dryer (see the first DNA extraction step below), and they can then be stored at **-20 °C** for several months.

3 In addition to the 2 mL screw-cap tube used to store the original sample, two clean labelled 1.5 mL microcentrifuge tubes are needed per sample.

4 If bulk beads are being used for bead beating, weigh 0.5 g aliquots into sterile microcentrifuge tubes, to be added to the original sample tube at a later stage.

Beads can also be added to 2 mL screw cap tubes prior to sample collection, or bead beating tubes with pre-aliquoted beads can be purchased instead of bulk beads, to reduce handling and possible contamination.

5 Pre-heat water bath to **37 °C** and an incubation oven with shaker or rotating rack to **55 °C** (can also use a second water bath or heat block that fits 2 mL cryotubes).

This incubation step is optimal if it includes rotating or gentle shaking. Where such equipment is not available, periodic manual mixing (e.g. every 10 minutes) is recommended.

6 Pre-heat another water bath to **65 °C** , and warm the stock solutions of 5 M NaCl and 10% CTAB.

If only one water bath is available, the same one can be used for the **37 °C** and **65 °C** steps . Increase

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temperature to 65 °C immediately after the 37 °C incubation.

10% CTAB stock solution often crystallises when stored at room temperature. If this has occurred, pre-heating to 65 °C is sufficient to dissolve the crystals. Mix by inversion before use.

7 Chill centrifuge to 4 °C (with rotor that can take 2 mL tubes).

8 Prepare fresh 70% ethanol and chill in ice bucket.

Making the 70% ethanol fresh will ensure the ethanol concentration is accurate. Old solutions of 70% ethanol likely have less ethanol than expected due to the higher vapour pressure of ethanol (compared with water).

DNA extraction: Cell lysis 1h 40m

9 Remove sample tubes from -80 °C freezer. Immediately add 570 µl lysis buffer. Vortex briefly, and either freeze in liquid nitrogen OR return to -80 °C freezer (freeze/thaw cycle) for a minimum of one hour (can be stored overnight or longer).

This freeze/thaw cycle is good for lysing difficult cell types (e.g. spores).

Lysis buffer can also be added immediately after the samples are removed from the freeze dryer, as described in step 2.

10 Remove from liquid nitrogen or freezer, thaw briefly in pre-heated 37 °C water bath. *[Optional]* Add pre-weighed beads and process on Qiagen TissueLyser II bead-beater for 40 s at 30 Hz (or equivalent on other models).

Bead beating can increase yield, but can also cause DNA shearing. It is suitable for most amplicon sequencing approaches (e.g. 16S / 18S rRNA). However, If high molecular weight DNA is required for downstream applications, for example in whole genome/metagenome sequencing, skip this bead beating step and go straight to step 11.

11 Add 1 µl Ready-Lyse stock per tube, mix well by inversion. Incubate at 37 °C for 00:30:00 30m with frequent mixing by inversion, to allow lysozyme to work.

Lysozyme is an enzyme that targets the peptidoglycan component of the bacterial cell wall. If bacteria are not the target organisms (e.g. for DNA extraction from animal tissue for genome sequencing), this step is not necessary and can be skipped.

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12 Add **30 µl** SDS stock (10%) and **3 µl** proteinase K stock per tube. Incubate at **55 °C** for **01:00:00** with frequent mixing (shaking or rotating incubator, or mixing by hand if using water bath or heat block). 1h

13 Move tubes to **65 °C** water bath. Add **120 µl** pre-warmed 5 M NaCl, mix well by inversion.

14 Add **96 µl** pre-warmed CTAB stock to each tube, mix by inversion and incubate at **65 °C** for **00:10:00**, with frequent mixing by inversion. 10m

DNA purification: Chloroform washes 10m

15 Remove from water bath. In fume hood, add **800 µl** chloroform:isoaamyl alcohol (or however much fits in the tube) and vortex for 5 seconds to form an emulsion.

If polycarbonate filters were used for sampling, they will dissolve in the chloroform.

16 Centrifuge **14000 x g, 4°C, 00:05:00** . From this point onwards, keep tubes chilled on ice. 5m

17 Remove top aqueous layer (approx. **700 µl**) into clean 1.5 mL tube. Do not disturb the interface.

18 Perform a second chloroform wash: add **700 µl** chl:iaa, vortex 5 seconds, then centrifuge **21000 x g, 4°C, 00:05:00** . 5m

This second chloroform wash results in a much cleaner DNA extract. It can be skipped if DNA purity is not important for downstream applications.

Centrifugation from here onwards should be carried out at the maximum speed possible on the available chilled benchtop centrifuge. Newer models can reach **21000 x g** . However, older centrifuge models might have lower maximum speeds (e.g. **14000 x g**). This shouldn't be a problem overall, but more care should be taken not to disturb any interface material between the chloroform and aqueous layers, and yields at the DNA precipitation step might be slightly lower.

19 With a 100 uL pipette, move the top aqueous layer to clean 1.5 mL tube. Record the total volume obtained from each tube. Be very careful not to move any chloroform or to disturb the interface.

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The top aqueous layer contains the DNA. The bottom layer is chloroform, and must be disposed of as chlorinated solvent waste.

DNA precipitation: Isopropanol 2h 55m

20 Add **1 µl** linear polyacrylamide (LPA) solution to each tube, vortex briefly to disperse.

LPA is a synthetic carrier that aids in the precipitation of very dilute DNA solutions.

21 For each tube, using the volume of aqueous layer recorded at step 19, calculate the amount of isopropanol to be added: (vol. isopropanol) = 0.7 * (vol. aqueous layer).

Precipitate the DNA by adding this volume of isopropanol to each tube (e.g. to 600 uL aqueous layer, add 420 uL isopropanol). Mix well by inversion.

22 Incubate in the dark at room temperature for at least **01:00:00** (preferably 2 hours). Precipitation can also be done overnight in the fridge at **4 °C** . 1h

23 Centrifuge at maximum speed **21000 x g, 4°C, 00:30:00** to pellet the DNA. 30m

Orient all tubes carefully and consistently in the centrifuge, e.g. with hinges facing outward, so that pellets are in the same place in each tube.

If the available centrifuge cannot reach 21000 x g, then 14 000 x g is sufficient but the time can be extended by 10 minutes or more if yields are expected to be low.

24 Remove the supernatant by decanting and/or pipetting. Be careful not to disturb the pellet.

Isopropanol pellets are usually clear, small, and can be difficult to see. They tend to turn white during later steps, when washed with ethanol.

Do not be alarmed if a pellet is observed in negative controls. LPA can sometimes form a visible pellet (but not always).

Pellets with some colouration (often brown/black) suggest impurities have precipitated along with the DNA (e.g. humics). These often do not cause problems, but can be removed later with a clean-up kit.

25 Wash pellets with **400 µl** ice-cold 70% ethanol. Rotate tubes so that ethanol washes all surfaces. Centrifuge at maximum speed **21000 x g, 4°C, 00:10:00** . 10m

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26 Carefully remove ethanol. Use a fine small-volume pipette tip to remove any small drops remaining in the bottom of the tube. Air-dry the pellets for **00:05:00** . 5m

Do not over-dry the pellets, as this will make them very difficult to resuspend.

27 Resuspend DNA in molecular-grade water, TE buffer, or Tris buffer. Mix gently by flicking the tube. Do not vortex or pipette up and down, as this will break long DNA strands and lead to degradation. If pellets do not resuspend easily, leave them at **Room temperature** for **01:00:00** and/or incubate tubes in a water bath at **50 °C** for **00:10:00** . Store at **-20 °C** . 1h 10m

The exact volume used for resuspension depends on the expected DNA yield and the desired final concentration. A good starting point is **50 µl** .

Once extracts are obtained, the following can be assessed:
- DNA quantity: Quantification with a fluorometric method (e.g. Qubit, QuantiFluor)
- DNA integrity: Gel electrophoresis at 0.8-1.0% agarose concentration, at low voltage (3V/cm), with a suitable high molecular weight marker (e.g. Lambda/HindIII)
- DNA purity: Absorbance ratios (A_{260}/A_{280}, A_{260}/A_{230}) measured by spectrophotometry (e.g. Nanodrop) to assess the presence of non-DNA components such as proteins, solvents, humics, etc. *(note: Nanodrop should not be used for DNA quantification)*

For amplicon sequencing, DNA extracts can usually be used directly in the PCR, though if amplification fails, dilution or clean-up may be required. For whole genome/metagenome sequencing, an additional clean-up with a commercial kit is usually required to remove impurities. The most appropriate kit depends on the sample type. With difficult samples, especially those from crustaceans and fungi, we have had the most success with the Zymo Genomic DNA Clean & Concentrate column-based kit. Other clean-up approaches that worked with non-crustacean samples include Qiagen Genomic-tips and Agencourt AMPure beads.

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