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CTAB extraction of DNA and RNA of respiratory samples for microbial work

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1 Works for me dx.doi.org/10.17504/protocols.io.bf28jqhw

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ABSTRACT

This extraction method is modified from Griffiths et al 2000 Applied Environmental Microbiology 66(12): 5488–5491 and DeAngelis et al 2009 ISME Journal 3 pp 168-178.

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In comparison with DNA extraction kits, nucleic acid yields tend to be higher, though it is more laborious for larger numbers of samples.The extraction uses CTAB and Phenol:Chloroform:Isoamyl alcohol for lysis and the precipitation of nucleic acid is PEG based. An alternative to bead-beating is described, which should only be used for extracting DNA from cultured isolates. Bead-beating is avoided in this case in order to minimise shearing of DNA prior to genomic DNA sequencing.For all other samples use bead-beating is recommended.

This Protocol will cover the extraction of the following sample types;

Bacterial isolates
Upper respiratory tract swabs (nasal and throat swabs)
Upper respiratory tract lavage and aspirates (nasopharyngeal aspirate, endotracheal aspirate, nasal lavage, Mouth wash)
Saliva
Sputum (expectorated spontaneously produced, and induced sputum)
Upper and lower respiratory tract synthetic absorptive matrix (SAM) strips
Lower respiratory tract samples obtained via bronchoscopy - lung brushes, bronchoalveolar lavage and pleural fluid
Lung tissue – human or mouse
Lung biopsies
Cerebral spinal fluid (CSF)

This is a double extraction protocol, you are adding a second volume of Phenol:Chloroform:Isoamyl alcohol to each sample, precipitating two aqueous phases per sample and then recombining the resulting DNA pellets.This

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approach significantly increases the yield of DNA from samples and strains.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Cowman SA, James P, Wilson R, Cookson WOC, Moffatt MF, Loebinger MR (2018) Profiling mycobacterial communities in pulmonary nontuberculous mycobacterial disease. PloS one. https://doi.org/10.1371/journal. pone.0208018

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MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

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KEYWORDS

DNA, Extraction, respiratory, microbial, microbiome, metagenomics, Respiratory

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MATERIALS

NAME	CATALOG #	VENDOR
Phenol:Chloroform:Isoamyl alcohol (25:24:1) Saturated with 10 mM Tris, pH 8.0		
Hexadecyltrimethylammonium bromide	H6269	Sigma Aldrich
Ethanol (molecular biology grade, ≥99.8%)	51976-500ML-F	Sigma Aldrich
Sodium Chloride	S9888	Sigma
Chloroform:Isoamyl alcohol 24:1	C0549	Sigma
lysing matrix E	116914050	
Phase Lock Gel Separation tube Heavy QuantaBio	733-2478	VWR International
FastPrep-24 Homogenizer	116004500	MP Biomedicals
Linear Polyacrylamide	56575-1ml	Sigma Aldrich
Aluminum ammonium sulfate	402816	Sigma Aldrich
Polyethylene glycol 6000	8074911000	Sigma Aldrich
0.5ml Plain Skirted Tube	E1405-2142	StarLab

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STEPS MATERIALS

NAME	CATALOG #	VENDOR
0.5ml Plain Skirted Tube	E1405-2142	StarLab
Phase Lock Gel Separation tube Heavy QuantaBio	733-2478	VWR International

SAFETY WARNINGS

- Informal training by a competent person before task can be undertaken unsupervised
- Appropriate PPE must be worn at all time
- Recommend doubling up nitrile gloves for handling phenol
- Work conducted at Containment Level 2
- Primary samples may be handled, but phenol and chloroform and to a lesser extent CTAB are the greater risks, so perform**work in the fume cupboard**, rather than the biological safety cabinet. Do not handle samples that are suspected to contain pathogens above ACDP category 2.

- Ensure correct waste disposal procedures are used. Waste solvents should be collected in labelled glass waste.
- The CTAB buffer cannot be disposed of down the sink as it is an environmental hazard. Collect waste solution as above.
- Note, when pipetting solvents, pre-wet the pipette tip by very gently pipetting up and down.This saturates the headspace within the tip with the volatile solvent and prevents vapour pressure from causing the tip to drip excessively when pipetting.
- Sealed buckets/rotors should be used for centrifugation steps
- Sample spillages should be disinfected with 70% ethanol followed by cleaning with Surfanios (Biolab, 059840)

Ensure clear and appropriate labelling of all stored samples

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This is a double extraction protocol, you are adding a second volume of Phenol:Chloroform:Isoamyl alcohol to each sample, precipitating two aqueous phases per sample and then recombining the resulting DNA pellets.This approach significantly increases the yield of DNA from samples and strains.

BEFORE STARTING

Prepare reagents:

CTAB extraction buffer:
1Part: 10% (w/v) CTAB (hexadecyltrimethylammonium bromide) in 1M NaCl
1 part: 0.5 M phosphate buffer (pH 8.0) in 1 M NaCl

Prepare **[M]1 Molarity (M)** NaCl in milliQ water.

Use the **[M]1 Molarity (M)** NaCl solution you have prepared in place of water for preparing the 10 % CTAB stock solution.
Prepare the phosphate buffer at pH 8.0.
- Prepare **[M]1 Molarity (M)** NaH2PO4 (Monobasic phosphate) and **[M]1 Molarity (M)** Na2HPO4 (Dibasic phosphate) stocks using **[M]1 Molarity (M)** NaCl in place of water.They may require gentle heating and stirring in order to dissolve.
- Combine **15.9 mL** of monobasic phosphate with **284.1 mL** of dibasic phosphate and make up to **600 mL** with **[M]1 Molarity (M)** NaCl to achieve pH 8.0
Combine the phosphate buffer and CTAB solution 1:1 to complete the CTAB extraction buffer
Sterilise by autoclaving

PEG/NaCl precipitation solution:
30 % (w/v) polyethylene glycol 6000 in **[M]1.6 Molarity (M)** NaCl
Sterilise by autoclaving

0.1M Aluminum ammonium sulphate:
[M]0.1 Molarity (M) Aluminum ammonium sulfate (AlNH4(SO4)2.12H20, Sigma 402816)
Filter-sterilize through 0.2mm filter

Before you start

1 Add working stock of Phenol:Chloroform:Isoamyl (P:C:I (25:24:1)) alcohol to a **50 mL** Falcon tubes in the fume cupboard.

Use a 10ml pippette to remove required volume of P:C:I from below the the top buffered layer in the stock

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bottle.
*For a new bottle ensure the buffer has been added and allowed to settle prior to starting the extraction.

2 Add working stock of Chloroform:Isoamyl (C:I (24:1)) alcohol to a **50 mL** Falcon tubes in the fume cupboard.

3 Pre-spin the 2 Phase-lock gel tubes (**2 mL** Hard gel) per samples .Phase lock gel should be pelleted at the bottom of each tube (often on the sides when they arrive from manufacturer). Centrifuge at 16,000 x g for **00:05:00** .

Phase Lock Gel Separation tube Heavy QuantaBio
by VWR International
Catalog #: 733-2478

4 Add **1 µl** of linear polyacrylamide (LPA) to **2 mL** eppendorfs, 2 per sample.This is a carrier and precipitates along with DNA increasing the yield. Unlike glycogen which can also be used it does not affect later sample use.

Extraction

5 Add **50 µl** aluminium ammonium sulfate to each Lysing Matrix E (LME) tube

It is helpful to write the sample numbers on the lid and side of the LME tubes as the beadbeater can rub the numbers off.

6 Aseptically transfer sample to the LME tube and add 500ul of CTAB to the LME tube and incubate for **00:15:00** .

Bacterial Isolates

a. Grow up bacterial isolates overnight in appropriate culture broth

b. Spin down **2 mL** of broth for **00:10:00** at 16,000 x g

c. In a safety cabinet add 500ul of CTAB and resuspend the pellet. Incubate at room temperature for **00:15:00** .

d. Bacteria and CTAB can be stored frozen prior to extraction or extracted after **00:15:00** incubation.**No additional CTAB is required**.

Upper respiratory tract swabs (nasal and throat swabs)

a. Prepare 1 sterile spin baskets in a **1.5 mL** trefflab tube per sample.

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b. 1 pair of autoclaved scissors will be required per swab to transfer swab tips into bead beating tubes
c. Keep swabs on ice until transferred into LME tubes
d. Aseptically transfer swab tips into the LME tubes using sterile scissors.
e. Add **500 µl** of CTAB to the LME tube and incubate for **00:15:00** .

Upper respiratory tract lavage and aspirates (nasopharyngeal aspirate, endotracheal aspirate, nasal lavage)

a. Spin a minimum volume of **2 mL** , optimal volume 5mls of lavage fluid for **00:20:00** at full speed
b. Carefully resuspend pellet in **500 µl** CTAB extraction buffer and transfer to LME tube.

NOTE: For saliva or mouth wash samples and sputum samples pre-alequoting using wide bore tips prior to storage is recommended.

Saliva or Mouth wash samples

a. Transfer a known volume of no more than **300 µl** into a LME tube.

Sputum (expectorated spontaneously produced, and induced sputum)

a. Transfer a known volume of no more than **300 µg** or individual sputum plug, into a LME tube.

Upper and lower respiratory tract synthetic absorptive matrix (SAM) strips

a. 1 pair of autoclaved scissors will be required per SAM to transfer SAM into LME tubes
b. Keep SAMs on ice until transferred into LME tubes

Lung brushes

a. Keep lung brushes on ice until transferred to LME tubes

Bronchoalveolar lavage or Pleural fluid

a. Spin a minimum volume of **2 mL** , optimal volume **5 mL** of lavage fluid for **00:20:00** at full speed
b. Carefully resuspend pellet in **500 µl** CTAB extraction buffer before transfering to LME tube.**NO further CTAB is required**

Lung tissue- Human or Mouse

a. Keep tissue on ice until transferred to LME tubes

Lung biopsies

a. Keep tissue on ice until transferred to LME tubes.

CSF

a.Maximise and standardise sample volume across the study.
b.Spin fluid for **00:20:00** at 16,000 x g
c.Carefully suspend pellet in **500 µl** CTAB extraction buffer before transferring to LME tube.**NO further CTAB is required**

Transfer of patient samples should be performed in a class 2 safety hood.

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7 Moving to a fume cupboard, carefully and immediately add **500 µl** of P:C:I (25:24:1).

Pre-wet the pipette to avoid drips due to vapour pressure in tip.

Perform this step in a fume hood

Wear double gloves when handling P:C:I (25:24:1).Should a small spillage occur, outer gloves can then be disposed of without risk.

DO NOT leave tubes with P:C:I (25:24:1) for extended periods, it can degrade the plastic

8 Transfer to the bead-beater and beat using the pre-programmed CTAB setting for **00:01:00** . Return tubes to ice immediately after beating.

CTAB Bead beating Program:

Speed: 5.5m/sec
Adapter: Quickprep
Time: 60 sec
Lysing Matrix: E
Quantity: 1ml
Cycles: 1
Pause time: 300sec

Ensure the lids of the LME tubes are securely fastened with no beads in the seal, and that the tubes are labelled on the top and on the side as the

9 Centrifuge LME tubes at 16,000 x g for **00:05:00** .

10 Transfer all liquid to phase lock gel tube and keep the LME tube on ice.

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Phase lock tubes should have been pre-spun. Phase lock gel should be pelleted at the bottom of each tube (often on the sides when they arrive from manufacturer). Centrifuge at 16,000 x g for **00:05:00**

11 Centrifuge the phase lock tube at 16,000 x g for **00:05:00** at **4 °C** .

Gel will form a barrier between the aqueous and P:C:I (25:24:1)/C:I (24:1) phases.

12 Add 1 volume of C:I (24:1) to each phase lock gel tube, shake briefly to mix. Centrifuge at 16,000 x g for **00:05:00** at **4 °C** .

If barrier does not form, extend centrifugation time.

13 **Second extraction**; In the fume hood, add **50 µl** of Aluminium ammonium sulphate, **500 µl** of CTAB extraction buffer and **500 µl** of P:C:I (25:24:1) to each bead beating tube.

14 Repeat steps 8 to 13 then continue with the precipitation step below.

Precipitation

15 Transfer the aqueous phase from each tube to the pre-prepared eppendorfs with **1 µl** of LPA in them. As the solvent is locked beneath the phase lock gel, you should be able to pour this. You may need 2 eppendorfs per sample to fit allow room for PEG.

16 Add 2 volumes of PEG/NaCl solution and mix well (solution is viscous). Leave overnight in the fridge at **4 °C** to precipitate.

If you are in a hurry it can be left for 2 hours but this will reduce the yield.

17 Centrifuge all eppendorfs at 16,000 x g for **00:20:00** at **4 °C** .

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18 Carefully aspirate the PEG/NaCl solution from the pellets.

Pellets are usually large and translucent. They tend to be fairly uniform in size as the LPA has also precipitated.

19 Wash pellets with **500 µl** ice-cold 70 % ethanol to remove any precipitated salts and centrifuge at 16,000 x g for **00:05:00** .

20 Repeat the wash twice with **200 µl** ice-cold 70 % ethanol.

21 After briefly air drying the pellet for about 5 minutes, resuspend in **30 µl** low EDTA TE and combine the resuspended total nucleic acid from each sample into a single tube (**60 µl** total per sample). For sputum samples (or other high biomass samples) you may need to increase this volume to **50 µl** per tube (**100 µl** per sample).

We do not recommend storing DNA in cryo tubes as you are unable to spin them down.
Recommended tubes: Tethered O ring, sterile tubes E1405-2142, star labs

0.5ml Plain Skirted Tube
by StarLab
Catalog #: E1405-2142

22 This extract can be stored at **-20 °C** or **-80 °C** until required.

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