Laboratory for Environmental Pathogens Research
Department of Environmental Sciences
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DNA extraction from water: 50-50-50 buffer-chloroform/phenol method

Materials

Extraction buffer (pH 8.0)[*]: 50 mM NaCl
50 mM Tris-HCl, pH 7.6
50 mM EDTA
5% SDS
(autoclave to sterilize)

Dithiothrietol 1 M[†]
Phenol (Tris-saturated), pH 8 (keep in refrigerator)
Chloroform:isoamyl alcohol (24:1)
Choroform
Sodium acetate solution 3 M (sterile)
Isopropanol
Ice-cold 70% EtOH (filter sterilized, 0.2 μm)
10% PVPP solution (sterile)
Microcentrifuge tubes (sterile)
For each sample: Three-2 ml tubes with locking lid
One-1.5 ml tube
Milipore filtration unit
Vacuum manifold
Sterile forceps
0.45 μm polycarbonate membrane filter with absorbent pad

- Wear gloves throughout the entire protocol.
- Do not cross-contaminate your samples or the solutions. Be aware of your pipette tip.
- Work clean, either on fresh blue bench paper, in the hood, or on a freshly ethanol treated bench top.
- Perform all centrifugations with the hinge of the tube pointing "up".
- Do not use a vortex at any point in this protocol unless specified.

All volumes are approximate unless otherwise noted. Some reagent volumes might need to be adjusted to account for sample characteristics.

The protocol in brief

You will isolate nucleic acids from bacteria in your water sample by first concentrating the bacteria onto a membrane. Then you will directly extract nucleic acids from the

[*] For low biomass/DNA samples, increase the buffer pH to 9.5.
[†] Use for difficult to extract samples

concentrated bacteria by bead beating. The nucleic acids will be purified by phenol/chloroform cleanup followed by precipitation by centrifugation in isopropanol.

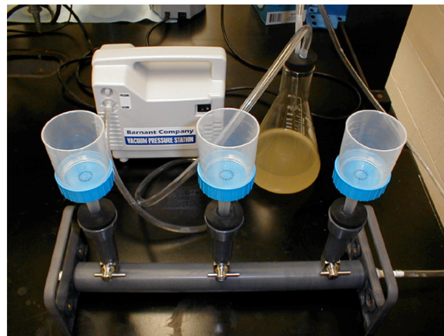
A. Filtering water to concentrate bacteria onto a membrane

1. Sterilize forceps by soaking in 70% ethanol and passing through a flame. The alcohol will kill any bacteria on the glass surface and the flame will burn-off the alcohol. With sterile forceps, remove a filter membrane (0.45 μm) and absorbent pad from plastic wrapping and place it onto the base of a Millipore disposable filtration unit. Be sure to place the pad-side down with the filter facing up. Snap the filter cup onto the base.

2. Place the filtration unit onto a vacuum manifold. Open all of the valves that control flow to vacuum ports that have filtration units attached. Close all others.

3. Place an appropriate volume of sample into the cup. The filter will hold 100 ml of water, but continue to add water following each filtration (below) in order to filter as much water as possible. Be sure to note exactly how much water was filtered.

4. Hook up the tubing from the vacuum pump to the manifold via the back-flow preventor. Turn the pump on. The water will be sucked through the filter membrane while cells present in the water will be trapped on the membrane.



The vacuum manifold (gray) with three filter units attached. A vacuum pump pulls solutions through the filters and collects the filtrate in a backflow preventor made from an Erlenmeyer flask.

5. Close the valves after each water sample has completely passed through each membrane to increase the vacuum suction to the remaining samples. Turn off the pump when each of the water samples has passed through the membranes.

6. Open the valves to release any remaining suction. Remove the filtration units from the vacuum manifold and remove the filtration cup from that base without disturbing the membrane.

7. With sterile forceps, carefully separate the membrane from the pad/base and place (bacteria-side "up") into an empty, sterile Petri dish.

8. Using flame-sterilized scissors, cut the membrane into small (1 cm^2) pieces. The DNA will be extracted from the bacteria trapped on these membrane pieces.

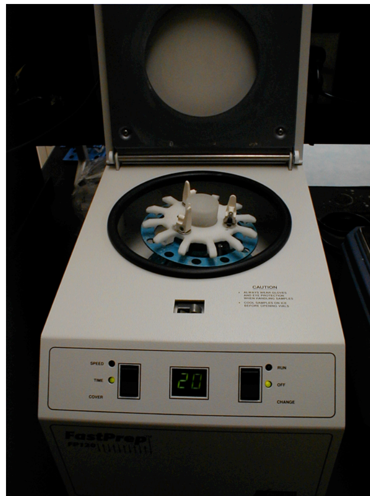
B. Nucleic acid extraction

9. Add 1.00 ml extraction buffer and 6-8 membrane pieces to a 2 ml microcentrifuge tube (with locking lid) containing 0.4 - 0.5 ml of glass beads (0.10 - 0.11 mm diameter).

10. Add exactly 1 μl of 1 M dithiothreitol into the lid of each sample, mix by vortexing for 2-3 seconds.

11. Shake with Fast-Prep instrument for **30** seconds at **5.5 ms^{-1}**.

    11a. To avoid leaks, be sure to remove any soil from the upper, inside area of the tube. Soil that remains there will cause a breach in the tube's seal.



A bead-beater rapidly shakes microcentrifuge tubes that contain sample and glass beads to lyse cells and release DNA.

12. Centrifuge for three minutes at 14,000 x g

C. Nucleic acid purification

13. Decant supernatant into fresh 2 ml tube. This solution contains the DNA. There is no need to pipette the supernatant. To save time, carefully <u>pour</u> the supernatant into a fresh tube. Discard the used tubes.

    13a. If you are extracting several samples for pooling, combine aqueous phase samples into a 15 ml falcon tube and proceed with the extraction in bulk until the isopropanol precipitation (step 8), at which time samples can be separated.

14. Determine the volume of your extract. This volume should be approximately 900 μl. Add half of this volume of both phenol and chloroform/isoamyl alcohol. Vortex until an emulsion forms and the solution appears milky (5-10 s). Centrifuge for 3 minutes at 14,000 x g or until phases are well separated. The aqueous phase containing the DNA will be the upper phase. With a sterile pipette tip, transfer the aqueous phase to a new 2 ml tube.

15. Extract again with an equal volume of chloroform (not chloroform/isoamyl alcohol) as above. Centrifuge as above, then transfer aqueous phase to a new 1.5 ml tube.

D. Nucleic acid precipitation

16. Determine the volume of your extract. Add exactly 0.1 volumes of 3 M sodium acetate solution and exactly 0.7 volumes of isopropanol. Mix well by inverting the tube several times. Do not vortex.

17. Precipitate the DNA by centrifugation at 14,000 x g for 30 minutes in the refrigerated centrifuge (10° C, tube hinges pointing up).

    17a. The appearance of the resulting pellets might range from transparent to quite dark in color, depending on the amount of organic acids co-extracted with the DNA.

18. Carefully discard the supernatant by aspirating the isopropanol. Isopropanol-precipitated pellets may detach from the side of the tube, so be careful not to loosen and/or dislodge the pellet. At this point, low biomass/DNA samples may be resuspended in ca. 100 ml of water, pooled, and re-precipitated in order to concentrate them to a manageable concentration.

19. Wash the pellet by adding 0.5 ml ice cold 70% EtOH and inverting the tube gently. Be sure that the EtOH contacts all surfaces inside the tube. Re-pellet the DNA again with centrifugation for 5 min. Optimization of this protocol has shown that centrifugation at cool temperatures ($10\text{-}15^{o}$ C) will result in better pellet formation and stability. Thus, the pellets are larger (containing more DNA) and will stick to the sides of the tube, which makes aspirating the alcohol easier. Remove EtOH as above being careful not to aspirate the pellet. Allow pellet to dry for 2-5 minutes.

E. Nucleic acid resuspension and final cleanup

20. Resuspend the pellet by adding exactly 50 μl of DNase/RNase-free water and mixing by flicking the tube with your finger until the pellet dissolves. Do not use nanopure or DI water for this purpose; these water sources are not clean enough.

21. Clean DNA with PVPP columns or a commercial kit if;

1. Further cleanup of contaminants is necessary.

2. You need to pool DNA samples from like samples. This will allow you to combine several aliquots of DNA and concentrate them while removing residual proteins and humic extracts.

PVPP cleanup (optional)

1. Add 300 ml of sterile 10% PVPP solution to an empty spin column. Place the spin column in a sterile 1.5 ml microcentrifuge tube.

2. Centrifuge at 14,000 x g for 1 minute. Empty the catch tube and repeat the centrifugation to dry the PVPP matrix. Place the spin column in a sterile 1.5 ml tube.

3. Increase the nucleic acid solution volume to at least 100 μl by adding DNase/RNase-free water. Add the entire volume to the spin column and centrifuge for 1 minute at 14,000 x g.

4. Repeat step 3 if necessary.

5. If the resulting DNA concentration is too low for your application, then reprecipitate with isopropanol as described above and suspend the resulting pellet in a smaller volume of sterile water.

Store your nucleic acid samples in the freezer (-20 ^{o}C), properly labeled. Do not assume that you will remember the extraction date, sample characteristics, etc. Write all of this information down in your lab book and mark your sample tubes accordingly.