

Chapter 2

Extraction of Total DNA and RNA from Marine Filter Samples and Generation of a cDNA as Universal Template for Marker Gene Studies

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Abstract

Microbial communities play an important role in marine ecosystem processes. Although the number of studies targeting marker genes such as the 16S rRNA gene has been increased in the last few years, the vast majority of marine diversity is rather unexplored. Moreover, most studies focused on the entire bacterial community and thus disregarded active microbial community players. Here, we describe a detailed protocol for the simultaneous extraction of DNA and RNA from marine water samples and for the generation of cDNA from the isolated RNA which can be used as a universal template in various marker gene studies.

Key words Metagenomics, Metatranscriptomics, Marker gene studies, Microbial diversity, Microbial functions

1 Introduction

Sequencing of marker genes has been widely used for the investigation of microbial communities in many environments including water [1] or microbial biofilms [2]. However, the vast majority of investigations focused on assessing entire community structures by 16S rRNA gene analysis and thus did not consider the active microbial community members. In the last few years, RNA-based studies have received more attention. These studies provided first insights into community structure and diversity of the potentially active microbes and their functions (for example [1, 3–5]).

Here, we describe a standard protocol for the simultaneous extraction of DNA and RNA from marine water samples. The extraction is based on the protocol described by Weinbauer et al. [6]. It is

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a combined mechanical and chemical extraction method utilizing a pH shift for the simultaneous extraction of RNA and DNA from individual membrane filters. Purified DNA-free RNA is subsequently converted to cDNA which can be used as a universal template in subsequent marker gene studies or for direct sequencing. The protocol has been already applied to investigate the response of marine archaeal and bacterial communities in the German Bight towards phytoplankton spring blooms [1, 4, 7]. Here, the cDNA served as template in PCRs targeting the archaeal and bacterial 16S rRNA transcripts. The provided method of cDNA generation can be applied to samples collected in a wide range of environments as long as high-quality environmental RNA is available.

2 Materials

Prepare all solutions using diethylpyrocarbonate (DEPC)-treated water and analytical grade reagents. For DEPC treatment, add 1 mL DEPC to 1 L ultrapure water. Stir for at least 1 h and remove residual DEPC by autoclaving at 121 °C for 20 min. Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1 Bacterioplankton Samples

The basis for the simultaneous DNA and RNA extraction are membrane filter samples obtained as follows: seawater samples are prefiltered through a 10- μm -mesh-size nylon net and a precombusted (4 h at 450 °C) 47 mm-diameter glass fiber filter (Whatman[®] GF/D; Whatman, Maidstone, UK). The free-living bacterioplankton is subsequently harvested by filtration of 1 L prefiltered seawater through a filter sandwich consisting of a glass fiber filter (Whatman[®] GF/F) and a 47-mm-diameter (pore-size 0.2 μm) polycarbonate filter (Nuclepore[®], Whatman).

2.2 Simultaneous DNA and RNA Extraction

1. Approximately 2 g of each 2 and 3 mm precombusted (4 h at 450 °C) glass beads (Carl Roth, Karlsruhe, Germany).
2. Sterile scissors and forceps.
3. Extraction buffer: 50 mM Sodium acetate and 10 mM EDTA, pH 4.2. Add about 800 mL water to a 1-L graduated cylinder or a glass beaker. Add 4.1 g sodium acetate and 3.72 g EDTA (disodium salt). Mix and adjust pH with acetic acid. Fill up to 1 L with DEPC-treated water.
4. SLS-solution: *N*-lauroylsarcosine sodium salt solution, 20%, for molecular biology (Sigma-Aldrich, St. Louis, USA).
5. Buffer-saturated phenol: Roti[®]-Aqua-Phenol (Carl Roth, Karlsruhe, Germany). Supplemented with 8-hydroxyquinoline, final concentration 1 mg/mL (*see Note 1*).
6. A high-speed cell disrupter such as the FastPrep[®]-24 Instrument (MP Biomedicals, Eschwege, Germany).

7. Greiner tubes, volume 50 mL (Greiner Bio-One, Frickenhausen, Germany).
8. 1 M Tris-base buffer, pH 10.5: Add 121 g Tris base to 900 mL water, adjust with HCl and fill up to 1 L.
9. 3 M sodium acetate, pH 4.8: Add 24.61 sodium acetate to a small amount of water. Mix and adjust pH with acetic acid. Fill with water to a final volume of 100 mL.
10. 24:1 chloroform–isoamyl alcohol (Carl Roth, Karlsruhe, Germany).
11. Isopropanol.
12. 35 mg/mL glycogen (Pepqab, Erlangen, Germany).
13. 96–100% ethanol.
14. 80% ethanol.
15. 1× TE buffer: mix 10 mL 1 M Tris-base, pH 8 adjusted with HCl, and 10 mL 0.5 M EDTA, pH 8 adjusted with NaOH with 980 mL DEPC-treated water.

2.3 Purification of Extracted DNA and RNA

1. 10 mg/mL Thermo Scientific™ RNase A (Thermo Fisher Scientific, Waltham, USA).
2. PeqGold Cycle-Pure Kit (Pepqab, Erlangen, Germany).
3. DEPC-treated water.
4. RNeasy MiniKit (Qiagen, Hilden, Germany).
5. 80% ethanol.
6. β-mercaptoethanol (Carl Roth, Karlsruhe, Germany).
7. Thermo Scientific™ 2× RNA loading dye (Thermo Fisher Scientific, Waltham, USA).

2.4 DNA Digestion and Control PCR

1. Ambion™ TURBO DNA-free™ Kit (Thermo Fisher Scientific, Waltham, USA) (*see Note 2*).
2. Thermo Scientific™ *Taq* DNA polymerase, recombinant (1 U/μL), reaction buffer with (NH₄)₂SO₄ (10×) and 25 mM MgCl₂ (Thermo Fisher Scientific, Waltham, USA).
3. Thermo Scientific™ 10 mM dNTP Mix (Thermo Fisher Scientific, Waltham, USA).
4. Thermo Scientific™ Ribolock RNase Inhibitor (40 U/μL) (Thermo Fisher Scientific, Waltham, USA).
5. Ten micromolar solutions of each of the following oligonucleotides: 8F (5'-AGAGTTTGATCCTGGCTCAG-3') [8], 518R (5'-ATTACCGCGGCTGCTGG-3') [9], 1055F (5'-ATGGCTGTCGTCAGCT-3') [10] and 1378R (5'-CGGTGTGTA CAAGCCCCGGAACG-3') [11] (*see Note 3*).
6. DEPC-treated water.
7. Phenol–chloroform–isoamyl alcohol (Carl Roth, Karlsruhe, Germany).

2.5 First and Second Strand Synthesis

1. Random hexamer primers (Roche, Penzberg, Germany).
2. Invitrogen™ SuperScript® Double-Stranded cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA).
3. DEPC-treated water.

3 Methods

Carry out all procedures at room temperature unless otherwise indicated. Use filter tips. Autoclave solutions (except SLS, phenol, phenol–chloroform–isoamyl alcohol and chloroform–isoamyl alcohol) and microtubes twice before use to avoid contamination with DNases, RNases, or nucleic acids. Wear gloves while working and take proper laboratory safety measures, i.e., work under a hood when dealing with phenol. Make sure to wear safety glasses and protective gloves, since phenol is highly toxic and corrosive. Diligently follow all waste disposal regulations when disposing of waste materials. Please read the notes added at the end of this protocol carefully.

3.1 Simultaneous DNA and RNA Extraction from Marine Filter Samples

3.1.1 Co-extraction of DNA and RNA

1. To prepare the extraction mixture for one extraction, mix 7.5 mL extraction buffer with 0.2 mL 20% SLS-solution. Scale up as needed.
2. Place glass beads in a fresh 50 mL Greiner tube.
3. Use sterile scissor and forceps to cut the frozen filter sandwich in short pieces. Place the filter pieces in the Greiner tube.
4. Add 5 mL of the extraction mixture.
5. Add 5 mL of buffer-saturated phenol.
6. Vibrate the mixture with 4 m/s for 60 s using a FastPrep®-24 Instrument.
7. Centrifuge for 20 min at $7200 \times g$ and 4 °C. During centrifugation, the mixture separates into a lower phenol phase with glass beads, an interphase and an upper aqueous phase.
8. Transfer the upper aqueous phase containing the RNA to a fresh Greiner tube.
9. Add 2 mL of the extraction mixture and 2 mL of buffer-saturated phenol to the phenolic phase for repeated phenol extraction.
10. Mix thoroughly by vortexing the capped tube and centrifuge again for 20 min at $7200 \times g$ and 4 °C.
11. Transfer the upper aqueous phase to the already collected aqueous phase from **step 8**. The volume of the pooled aqueous phases is about 5 mL.
12. For DNA isolation, add 5 mL Tris-base to the phenolic phase and mix well. Store at 4 °C for at least 40 min but not longer than 3 h.
13. Continue with RNA isolation.

3.1.2 RNA Isolation

1. Add 0.1 volumes of 3 M sodium acetate to the Greiner tube containing the pooled aqueous phases from the extraction and phase separation procedure (*see* Subheading 3.1.1, **step 11**).
2. Add 5 mL of chloroform–isoamyl alcohol (*see* **Note 4**).
3. Mix vigorously by vortexing and centrifuge for 10 min at $9000\times g$ and 4 °C to separate the phases.
4. Transfer the aqueous phase to a fresh Greiner tube and repeat **steps 2 and 3**.
5. Transfer the aqueous phase to a fresh Greiner tube and add a 1/700 volume of glycogen (*see* **Note 5**).
6. Mix vigorously and add 1 volume of isopropanol to precipitate the RNA.
7. Mix vigorously and incubate samples at –20 °C overnight (*see* **Note 6**).
8. Continue with DNA isolation.

3.1.3 DNA Isolation

1. Mix the Greiner tube containing the extracted DNA vigorously from Subheading 3.1.1, **step 12** and centrifuge for 15 min at $2000\times g$ and 4 °C.
2. Transfer upper aqueous phase to a fresh Greiner tube.
3. Add 2 mL 1 M Tris-base to the lower phenolic phase and repeat mixing and centrifugation for 15 min at $2000\times g$ and 4 °C.
4. Transfer upper aqueous phase to the aqueous phase already collected in **step 2**. Add 5 mL of chloroform–isoamyl alcohol to the Greiner tube.
5. Mix by vortexing and centrifuge for 10 min at $9000\times g$ and 4 °C to separate the phases.
6. Transfer upper phase to a fresh Greiner tube and repeat **steps 4 and 5**.
7. Add 1/10 volume of 3 M sodium acetate and 1/700 volume of glycogen (*see* **Note 5**).
8. Mix vigorously. Add 2.5 volumes of ice-cold pure ethanol to precipitate the DNA.
9. Mix vigorously and incubate samples at –20 °C overnight (*see* **Note 6**).

3.1.4 Washing and Resuspension of RNA and DNA

1. Pellet the precipitated nucleic acids (*see* Subheading 3.1.2, **step 7** and Subheading 3.1.3, **step 9**) by centrifugation at maximum speed for 30 min and 4 °C.
2. Wash the pellets twice with 1 mL of ice-cold 80% ethanol. Centrifuge at maximum speed for 10 min and 4 °C.
3. Dry pellet for about 10 min at room temperature (*see* **Note 7**).
4. Dissolve the DNA or RNA in 200 μ L 1 \times TE buffer.

3.2 DNA and RNA Purification

3.2.1 Removal of Residual RNA from DNA Samples

1. Add 1 μL RNase A to the extracted DNA (*see* Subheading 3.1.4, **step 4**).
2. Incubate at 37 °C for 1 h.
3. Purify the DNA using the peqGold Cycle-Pure Kit.
4. Elute DNA with 100 μL pre-warmed DEPC-treated water.
5. Repeat DNA elution with 100 μL Tris buffer (supplied with the Kit).

3.2.2 RNA Purification with the Qiagen RNeasy MiniKit

1. Add 700 μL RLT buffer and 7 μL β -mercaptoethanol to the RNA from Subheading 3.1.4, **step 4**. Mix well.
2. Add 500 μL of 96–100% ethanol to the diluted RNA. Mix well. Do not centrifuge. Proceed immediately to **step 3**.
3. Transfer 700 μL of the sample to an RNeasy Mini spin column placed in a 2 mL collection tube (supplied). Close the lid gently, and centrifuge for 15 s at $>8000\times g$. Discard the flow-through.
4. Repeat **step 3** once.
5. Place the RNeasy Mini spin column in a new 2 mL collection tube (supplied).
6. Add 500 μL Buffer RPE (supplied) to the spin column.
7. Close the lid gently and centrifuge for 15 s at $>8000\times g$. Discard the flow-through.
8. Add 500 μL of 80% ethanol to the RNeasy Mini spin column. Close the lid gently, and centrifuge for 2 min at $8000\times g$.
9. Place the RNeasy Mini spin column in a new 2 mL collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the flow-through and collection tube.
10. Place the RNeasy Mini spin column in a new 1.5 mL collection tube (supplied). Elute RNA two times with 50 μL DEPC-treated H_2O (~ 95 μL eluate).
11. Mix 5 μL of the purified RNA with 5 μL 2 \times RNA loading dye and control the success of the RNA extraction and purification by agarose gel electrophoresis.

3.2.3 DNA Digestion

1. If nucleic acid solution concentration is higher than 200 ng/ μL , dilute with DEPC-treated water.
2. Add 1/10 volume of 10 \times TURBO DNase Buffer (supplied) to RNA sample.
3. Add 1/40 volume of Ribolock RNase Inhibitor (final concentration 1 U/ μL).
4. Add 1 μL TURBO DNase (2 U) per 10 μg of RNA (*see* **Note 2**).
5. Incubate at 37 °C for 30 min.

6. Add 0.5 μL TURBO DNase (1 U) for every 10 μg of RNA.
7. Incubate at 37 °C for additional 15 min.
8. Add 0.1 volumes of DNase Inactivation Reagent (supplied) and mix well.
9. Incubate at room temperature for 5 min. Mix occasionally.
10. Centrifuge at 10,000 $\times g$ for 1.5 min and transfer the RNA to a fresh tube.
11. Perform 16S rRNA control PCR (*see* Subheading 3.2.4). Repeat **steps 3–9** if necessary.
12. Add 1 volume of 25:24:1 phenol–chloroform–isoamyl alcohol and mix thoroughly.
13. Centrifuge at 14,000 $\times g$ and 4 °C for 5 min.
14. Carefully transfer the upper aqueous layer to a fresh tube.
15. Add an equal volume of chloroform–isoamyl alcohol (24:1) and mix thoroughly.
16. Centrifuge at 14,000 $\times g$ and 4 °C for 5 min.
17. Carefully transfer the upper aqueous layer to a new tube.
18. Add 1/10 volume of sodium acetate and 1 μL glycogen (10 mg/mL) and mix.
19. Add 2.5 volumes of ice-cold absolute ethanol and mix by vortexing.
20. Incubate overnight at –20 °C.
21. Centrifuge at 4 °C for 30 min at 14,000 $\times g$.
22. Remove the supernatant carefully, avoid to lose the pellet.
23. Overlay the pellet with 0.5 mL of ice-cold 70 % ethanol.
24. Centrifuge for 10 min at 14,000 $\times g$.
25. Remove the supernatant carefully; avoid to lose the pellet.
26. Dry the pellet at RT for 10 min to remove residual ethanol.
27. Dissolve the pellet in a small volume of DEPC-treated water (~12 μL per filter).

3.2.4 Control PCR for Residual DNA

1. Combine the following ingredients in a sterile DNA-free 0.2 mL PCR tube: 2.5 μL 10 \times *Taq* buffer, 1 μL dNTP mix, 2 μL 25 mM MgCl_2 , 1 μL of each of the four oligonucleotides, and 1 μL *Taq* DNA polymerase (1 U/ μL). Add up to a final volume of 24 μL with H_2O . The reaction mix can be scaled up as needed.
2. Add 1 μL of DNase-treated RNA from Subheading 3.2.3, **step 10** to the mixture.
3. Perform negative controls using the reaction mixture without template.

4. Use the following thermal cycling scheme: initial denaturation at 94 °C for 2 min, 28 cycles of denaturation at 94 °C for 1.5 min, annealing at 55 °C for 1 min, followed by extension at 72 °C for 40 s. Final extension at 72 °C for 10 min.
5. Control the success of the DNA digestion by running 5 µL of the PCR reaction on a 2% agarose gel.

3.3 First and Second Strand Synthesis

1. Add 1 µL of random hexamer primers to 10.5 µL of DNA-free RNA from Subheading 3.2.3, step 27.
2. Incubate at 70 °C for 10 min.
3. Quick-chill on ice.
4. Add in the following order: 4 µL first strand buffer, 0.5 µL Ribolock, 2 µL 100 mM DTT and 1 µL dNTP mixture.
5. Vortex gently and collect the reaction by brief centrifugation.
6. Incubate at 25 °C for 2 min to equilibrate the temperature.
7. Add 1 µL SuperScript™ II RT (200 U).
8. Incubate at 25 °C for 10 min.
9. Incubate at 45 °C for 1 h.
10. Place on ice.
11. On ice, add the following reagents in the order shown to the first-strand reaction tube: 94 µL DEPC-treated water, 30 µL second strand buffer (5×), 3 µL dNTPs, 0.5 µL *E. coli* DNA ligase (10 U/µL), 2 µL *E. coli* DNA polymerase I (10 U/µL), 0.5 µL *E. coli* RNase H (2 U/µL) (*see Note 8*).
12. Vortex gently to mix and incubate for 2 h at 16 °C. Do not allow the temperature to rise above 16 °C.
13. Add 1 µL (10 U) of T4 DNA Polymerase and continue to incubate at 16 °C for 5 min (*see Note 8*).
14. Place the tube on ice and add 10 µL of 0.5 M EDTA.
15. Purify with Bioline SureClean Plus Solution as recommended by the manufacturer (*see Note 9*).

4 Notes

1. 8-hydroxyquinoline works as an antioxidant. In addition, it has a helpful side effect: the phenol turns yellow. Thus, it simplifies the phase separation as the aqueous phase is colorless whereas the phenol phase is yellow.
2. DNases are sensitive to mechanical forces. Therefore, handle all solution containing a DNase carefully. Do not mix by vortexing.
3. The four oligonucleotides target two regions of the bacterial 16S rRNA gene. Hence, two PCR products are formed during

multiplex PCR (~500 bp and ~310 bp). If your desired PCR product is shorter than 300 bp, please use the same oligonucleotides you are planning to use in your marker gene study.

4. The chloroform–isoamyl alcohol is used to remove any residual phenol which is crucial for any downstream application.
5. Adding glycogen to any precipitation mix has two advantages. Firstly, the glycogen forms a visible pellet with the DNA. Secondly, the precipitation efficiency is increased.
6. DNA and RNA can be stored in precipitation mixtures for several months up to 1 or 2 years.
7. It is important not to let the pellet dry completely as this will greatly decrease its solubility.
8. The amounts of enzymes used in the second strand synthesis are halved compared to the manufacturer's protocol but sufficient for cDNA synthesis. Additional second strand buffer and SuperScript™ II RT can be ordered from the supplier.
9. A PEG-solution (20% PEG 8000, 2.5 M NaCl) can be used as an alternative to Bioline SureClean Plus Solution. Add 1 volume PEG-solution to the reaction mixture from Subheading 3.3, **step 15**. Incubate at room temperature for 15 min followed by centrifugation at $14,000\times g$. Wash the pellet with ethanol (80%) and centrifuge at $14,000\times g$ for 5 min. Repeat the washing step. Dry the pellet at room temperature for 10 min and resuspend the cDNA in 50 μ L DEPC-treated water or TE-buffer (1 \times).

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