Sample Collection

1. Rinse filter holders, tubing, and sample bottles with 10% hydrochloric acid (HCl), then ultrapure (e.g., Milli-Q or Nanopure) water before each use.
2. Place filter membranes in 25 mm polypropylene, in-line filter holders (Advantec). Three different pore sizes (a-c) will be used for most samples, one membrane per holder:
    a. 3 μm Polycarbonate, 25 mm diameter; Millipore
    b. 0.6 μm Polycarbonate, 25 mm diameter; Millipore
    c. 0.2 μm Supor, 25 mm diameter; Pall
3. Rinse sample bottles (5-L capacity polycarbonate) twice with sample then fill directly from the Niskin bottles
4. Thread pump tubing through peristaltic pump heads and place inlet end of pump tubing into the bottles.
5. Pump 100-200 ml of sample through the tubing (no filter attached) into the sink to rinse the lines.
6. Connect 3.0 μm filters in housings to the ends of the tubing and start pump on very slow speed to check lines and purge air if necessary (To purge air bubbles, loosen housing slightly and hold at an angle to let air escape then re-tighten the housing, be sure the filter remains properly seated!)
7. Attach the 0.6 μm filter housing onto the outlet of the 3.0 μm housing (repeat air purge if necessary), then attach the 0.2 μm filter housing to the outlet of the 0.6 μm housing (repeat air purge if necessary)
8. Attach drain tubing to the outlet of each filter stack and direct the tubing into a collection bottle.
9. Pump sample though the filters at 20 to 50 ml min^{-1} (or as a slow as practical)
10. For those samples for which the 0.02-0.2 μm fraction will be analyzed as well, set up another tubing line to sample from the filtrate collection bottle, rinse the line with 100-200 ml of filtrate by pumping, then place a 0.02 μm filter (Anotop, Whatman) on the outlet. Turn on pump (14 ml min^{-1}) and collect the filtrate (ca. 2 liters) in another collection carboy.
11. At the end of sample filtration remove the tubing inlet from the bottle and continue pumping to drive all the water from the line and the filter housing.
12. If filter housings cannot be pumped dry (e.g., filtration has slowed dramatically and lines and housings are still full of water), then remove housings from lines, connect a 30-60 cc syringe to the inlet and drive water out with air pressure by hand).
13. Record the volumes filtered by measuring the filtrate volumes (for samples filtered through a 0.02 μm filter, record volume of both the < 0.2 μm and the < 0.02 μm filtrates)
14. Place each filter membrane in a 2-ml, screw-cap, polypropylene centrifuge tube using forceps. Wrap the filters around the wall of the tube with the sample side facing toward the interior of the tube.
15. Place filters at -80 °C until all samples from the same batch are ready for further processing.

DNA Extraction

This protocol uses the reagents in the MasterPure DNA purification kit (Epicentre), which is based on a salting out protocol originally published by Miller et al. (1988). The protocol in the current kit form was described in Watson et al. (1998). Lysis is achieved by a combination of detergent, enzyme digestion, and heat. RNA is removed by enzymatic digestion, then protein-SDS complexes and cell debris are separated from the remaining DNA by "salting out" with the addition of ammonium acetate followed by centrifugation. DNA in the supernatant is then concentrated and separated from other soluble components by alcohol precipitation. A general overview of extraction and purification of nucleic acids can be found in Steward & Culley (2010). A detailed evaluation of various steps used in the extraction of nucleic acids from aquatic microorganisms is provided by Boström et al. (2004). An evaluation of nucleic acid extractions from Anotop aluminum oxide filters can be found in Mueller et al. (2014).

Membrane filters in tubes

1. Combine lysozyme to TES buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5) in sufficient volume for the number of samples to be extracted (200 U lysozyme and 450 μl TES buffer per sample)
2. Add 450 μl TES/Lysozyme solution to each sample and incubate 30 minutes at room temperature ensuring filter is thoroughly wetted with solution
3. Combine proteinase K and 2× Tissue & Cell Lysis Buffer in sufficient volume for the number of samples to be extracted (3 μl proteinase K and 450 μl buffer per sample).
4. Add 450 μl of lysis buffer/proteinase K to each tube and mix thoroughly
5. Mix the tubes with a vortex briefly then incubate at 65 °C for 1 hour on their sides in bottles attached to the rotisserie in a hybridization oven.
6. Cool extracts then add 3 μl of RNase solution to each tube. Mix with a vortex briefly then incubate at 37° (± 3 C°) for 30 min.
7. Remove filters from the extracts using a pipet tip as a pick (new tip for each sample). Discard the filter and save the extract
8. Chill extracts on ice, add 520 μl of MPC Protein Precipitation Reagent to each, mix again by brief vortex mixing and incubate for 10 min on ice
9. Centrifuge the tubes for 10 minutes at maximum speed (~12,000 to 18,000 ×g) in a refrigerated microcentrifuge (4 °C).
10. Transfer a portion of each supernatant (~1000 μl) to fresh 2 ml tubes (avoiding the pellet) and centrifuge again under the same conditions.
11. Transfer 800 μl of the supernatant (avoiding pellet if there is one) to fresh tubes and add one volume (800 μl) of ice-cold isopropanol to each, invert multiple times to mix thoroughly, then incubate on ice for 10 minutes. (Freeze the residual ~200 μl of extract as a back-up archive if desired).
12. Centrifuge samples for 20 minutes at maximum speed in a chilled microcentrifuge (4 °C).
13. Remove supernatants and discard, being careful to leave the DNA pellet.
14. Add 0.5 ml of ice-cold 70% ethanol and centrifuge again for 2 minutes at maximum speed.

15. Remove and discard the supernatant using extra care to avoid the pellet, which at this stage is hard to see does not adhere well to the wall of the tube. Although it may dislodge from the tube and move around, it should remain intact. Carefully pipet around it, rotating the tube as necessary to get it out of the way of your tip.
16. Repeat steps 14 and 15 once and take care to remove as much supernatant as possible to facilitate drying of the pellet.
17. Place uncapped tubes with pellets in a hot block (pre-warmed in a 65 °C oven) until any residual liquid has evaporated (1 to 10 minutes). Check tubes frequently to avoid excessive drying of the pellets, which can inhibit subsequent rehydration and solubilization of the DNA.
18. Resuspend pellets in 50 μl of nuclease-free water or Tris-EDTA buffer (Life Technologies) allowing them to incubate at room temperature for ~1 hour with periodic vortexing.
19. Dilute a small portion of the extract in nuclease-free water for subsequent PCR. Dilution of ≥ 10-fold will help eliminate PCR inhibition.
20. Store remainder of DNA extracts at -20 °C.

Anotop filters

1. Place sufficient TES/lysozyme buffer for the number of samples (500 μl per sample) into a *charging* syringe, then load ca. 500 μl of buffer into 3-ml capacity *injection* syringes (one syringe per sample), by connecting them tip-to-tip with a female-female luer adapter
2. Attach an Anotop filter *by its outlet* to the tip of each injection syringe using a female-female luer adapter and attach an empty 3-cc *aspiration* syringe to the filter inlet, then hold the assembly vertically with injection syringe on the bottom and inject 500 μl of TES/lysozyme into filter.
3. Incubate 30 min at RT.
4. While the samples are incubating, place sufficient lysis buffer/proteinase K for the number of samples (500 μl per sample) into a fresh charging syringe, then load ca. 500 μl of buffer into 3-ml capacity *transfer* syringes (one syringe per sample), by connecting them tip-to-tip with a female-female luer adapter.
5. After the 30 min lysozyme incubation, gently pull most or all of the lysozyme solution back into the injection syringe
6. Detach the injection syringe with its female-female luer adapter from the Anotop filter and transfer 500 μl of lysis buffer/proteinase K into each injection syringe from the transfer syringe (push back and forth to mix the solutions, then transfer completely to the injection syringe)
7. Reattach the injection syringe to the filter outlet and inject most of the lysis buffer mixture into the filter (excess will appear in the aspiration syringe).
8. Attach syringe-filter-syringe assembly to the rotisserie in the hybridization oven and incubate at 65 °C at maximum rotation speed.
9. After incubation, remove fluid from the filter by holding vertically with aspiration syringe on the bottom and gently drawing extract into the aspiration syringe.
10. Transfer the extract (~900 μl) to a microcentrifuge tube and proceed as described above for "Membranes filters in tubes" starting at **Step 6**.

References

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Miller SA, Dykes DD, Polesky HF (1988). A simple desalting procedure for extracting DNA from human nucleated cells. *Nucl Acids Res* 16: 1215.

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Steward GF, Culley AI (2010). Extraction and purification of nucleic acids from viruses. In: Wilhelm SW, Weinbauer MG, Suttle CA (eds). *Manual of Aquatic Viral Ecology*. American Society of Limnology and Oceanography: Waco, TX. pp 154-165.

Watson J, Schanke J, Grunenwald H, Meis R, Hoffman L, Lewandowska-Skarbek M *et al* (1998). A new method for DNA and RNA purification. *J Lig Assay* 21: 394-403.

PCR Amplification for Illumina Tag Sequencing

This protocol is based on that published by Caporaso et al. (2012) specifically for the high-throughput, multiplex sequencing on the Illumina platform of amplicons targeting the V4 region of the 16S rRNA gene. A summary of the protocol upon which the following information is based can be found online at: http://www.earthmicrobiome.org/emp-standard-protocols/16s/

Information on the Primers used for amplification:

The primers used for amplification include not only the sequences that target the one of the hypervariable regions of the 16S rRNA gene (V4), but additional nucleotides that serve as capture probes and primers for the Illumina sequencing reactions as well as linkers and spacers to join the regions together. In addition, one of the primers has a region 12 nucleotides long (the tag or bar code), which has a variable sequence. Every sample is amplified with a unique version of the bar code. In addition to sequencing an amplicon, one also sequences the bar code so one can determine from which sample a given sequence derives.

Illumina 515f primer construct

The forward primer sequence (515f) has four regions: 1) 5' Illumina adapter, 2) primer pad, 3) primer linker, and 4) 516f primer. The complete sequence is shown here with the regions delimited by brackets:

[AATGATACGGCGACCACCGAGATCTACAC] [TATGGTAATT] [GT] [GTGCCAGCMGCCGCGGTAA]

Illumina 806r primer construct

The reverse primer sequence (806r) has five regions: 1) Reverse complement of 3' Illumina adapter, 2) Golay Bar code (region marked by Xs), 3) primer pad, 4) primer linker, and 4) 806r primer. The complete sequence is shown here with the regions delimited by spaces:

[CAAGCAGAAGACGGCATACGAGAT] [XXXXXXXXXXXX] [AGTCAGTCAG] [CC] [GGACTACHVGGGTWTCTAAT]

Amplification Reaction Mix

Complete reagent recipe (master mix) for 1X PCR reaction	
*PCR Grade H_2O	13.0 μL
*Hot Master Mix	10.0 μL
*Forward Primer (10μM)	0.5 μL
Reverse Primer - tagged (10μM)	0.5 μL
Template DNA	1.0 μL
Total reaction volume	25.0 μL

*These reagents can be combined in proper ratio then add 23.5 μl per reaction. Reverse primer and template must be added individually. Reverse primer with unique tag is used for each sample.

Protocol

1. (Optional) Work in an enclosed cabinet equipped with a UV lamp to help minimize contamination from non-template DNA (Fig. A2). UV irradiate the cabinet and the PCR tubes with caps open for 20 min prior to starting to set up the reactions.
2. Thaw Master Mix, primers, and template while cabinet is being irradiated
3. In the cabinet, combine PCR-grade water (MoBio Laboratories, Inc.; Cat#1700-11) and Hot Master Mix (5 Prime; Cat# 2200410) in sufficient quantity for the total number of reactions to be run (i.e. combine 13 μl × n of water and 10 μl × n of Master Mix in a tube, where n is the number of samples) and mix thoroughly. Include one or two extra samples in the budget to account for minor pipetting errors.
4. Distribute 23 μl of the diluted master mix into each reaction tube.
5. Add 1 μl of bar-coded primer pairs (10 μM stock) to each reaction tube, ensuring that each sample receives a unique bar code.
6. Add 1 μl of template (for our samples, DNA extract will be diluted five-fold in PCR-grade water prior to using as a template in order to minimize inhibition of the reaction by other constituents in the extract). Total template in each reaction will vary but will be in on the order of 0.1 to 5 ng.
7. Subject the tubes to the following thermal cycling protocol:

    1. Initial Denaturation step at 94°C for 3 minutes
    2. Thirty-five cycles of:
       94°C 45 seconds
       50°C 60 seconds
       72°C 90 seconds
    3. Final Extension at 72°C 10 minutes
    4. Hold indefinitely at 4°C

Examine PCR products by agarose gel electrophoresis (protocol below) to check for yield and specificity of the reaction.

Three sequencing primers will be used later with this sample (after pooling all amplicons together in equal mass ratio and clean up of the pooled amplicons). These were 1) a forward read primer (Read 1), 2) a reverse read primer (Read 2) and 3) a primer to read the bar code (Index Sequence Primer):

Read 1 Sequencing primer:

Field description (space-delimited):

1. Forward primer pad
2. Forward primer linker
3. Forward primer

TATGGTAATT GT GTGCCAGCMGCCGCGGTAA

Read 2 Sequencing primer:

Field description (space-delimited):

1. Reverse primer pad
2. Reverse primer linker
3. Reverse primer

AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT

Index Sequence primer:

Field description (space-delimited):

1. Reverse complement of reverse primer
2. Reverse complement of reverse primer linker
3. Reverse complement of reverse primer pad

ATTAGAWACCCBDGTAGTCC GG CTGACTGACT

Reference

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N *et al* (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* **6:** 1621-1624.

Agarose Gel Electrophoresis

This protocol takes advantage of a low-ionic strength electrophoresis buffer which is cheaper and simpler to make than traditional TBE or TAE buffers, and allows one to use much higher voltages, thereby shortening the time needed for analysis (Brody et al. 2004; Brody & Kern 2004).

1. Melt molecular biology-grade agarose in electrophoresis running buffer (5 mM lithium borate buffer; Faster Better Media, LLC) by boiling in a microwave for a final concentration of 1% w/v. The gel rig we are using takes 70-80 ml of agarose.
2. Cool the molten agarose until comfortable to touch, then spike with fluorescent DNA stain (final concentration 1× SYBRSafe; Invitrogen).
3. After further cooling to near the gelling temperature, pour the agarose into a gel tray (with combs in place), which has been clamped in a casting frame.
4. Manually gimbal the casting frame to counteract the motion of the ship and keep the tray level while the gel solidifies.
5. After solidification, remove the combs, unclamp the ends of the casting tray, and fill the wells with running buffer.
6. Mix a 5× Loading Medium containing Orange G dye (Faster Better media, Inc.) 1:1 with water and place 5 μl spots of the diluted loading medium on a piece of Parafilm M (one spot per sample to be loaded).
7. Mix a portion each sample (typically 5 μl of the PCR reaction) and 1 to 4 μl of size marker (100 bp ladder; Invitrogen) with a spot of loading medium on the Parafilm, by pipetting up and down in the spot.
8. Pipet the sample/loading buffer mixture into the wells of the gel.
9. Place the gel in an electrophoresis tank and add running buffer to the reservoirs on either side until the buffer level just reaches the upper edge of the gel (if the gel is covered, motion of the ship creates turbulence that can mix some sample out of the wells). Place the cover on the tank and start electrophoresis at 200V for 1-2 minutes to allow sample to migrate out of the wells and into the gel.
10. Remove the lid, add more buffer to cover the gel (by about 3-5 mm), then place the lid back on and resume electrophoresis at 200 V for 10 to 15 minutes.
11. Examine gels on a UV transilluminator through a UV-blocking shield and image with camera.

References

Brody JR, Calhoun ES, Gallmeier E, Creavalle TD, Kern SE (2004). Ultra-fast high-resolution agarose electrophoresis of DNA and RNA using low-molarity conductive media. *Biotechniques* **37:** 598-602.

Brody JR, Kern SE (2004). History and principles of conductive media for standard DNA electrophoresis. *Anal Biochem* **333:** 1-13.

Pooling amplicons for sequencing

1. Measure the concentration of amplicon in each reaction to be sequenced by fluorescence assay (Qubit DNA assay)
2. Pool appropriate volumes of each sample so that all contribute equal mass to the pooled sample
3. Run the pooled PCR product on a gel
4. Excise the band from the gel and purify using the gel purification kit (MoBio). Elute the sample into water
5. Take an aliquot of the final product to quantify the DNA concentration by fluorescence (Qubit DNA assay)