

Lake Microbial Community Protocol (ARISA)

Revised 15 Feb 08 ALS

Lake Microbial Community Analysis Workflow- ARISA

I. Sample Collection

Needed: Autoclaved container for water sample
Hose or PVC integrated water column sampler
Cooler
Boat, lifevests, paddle
Notebook
Any physical/chemical water measurement equipment

1. Rinse sampler twice with lake water from sample location. Rinse autoclaved collection contained with lake water three times.
2. Collect sample to appropriate depth.
3. Place water sample into collection container. For ARISA, each replicate requires from 150 to 500 mL of lake water. Do not put your fingers into the water; try to keep it as “clean” as possible.
4. Place container into a cooler to maintain temperature of lake water until filtration. Sample must be filtered as soon as possible to preserve bacterial community.

II. Filtration

Needed: Vacuum pump
Vacuum manifold
Autoclaved filter funnels
0.2 um Whatman filter- 1 per replicate
EtOH
Forceps
1 cryovial per replicate

1. Set up the vacuum pump and manifold. Wear gloves from this part onward.
2. Place autoclaved yellow 500mL filter funnels onto manifold.
3. Sterilize forceps in EtOH, then rinse with diH2o. Using forceps, place one Whatman 0.2 um filter on inside funnel ring. Ensure funnel top has o-ring. Tighten funnel top onto filter.
4. Shake the sample water inside its container to mix, then rinse funnel with sample. Add appropriate sample volume (150 or 500 mL water- make note of how much volume was filtered) to funnel.
5. Apply vacuum. Allow to filter until all the liquid has pass through the filter. Release vacuum. Remove funnel top. Re-apply vacuum to dry the filter.
6. Sterilize forceps in EtOH. Using forceps, quickly and aseptically fold and transfer filter to cryovial. Sample can be frozen at -80C until Filter Preparation.

III. Filter Preparation for DNA extraction

Needed: Jar of 95% EtOH
1 razor blade per filter
1 sterile petri dish per two filters
forceps
2 mL screw-cap bead beater tubes (2 per filter)

1 ceramic bead (1 per bead beater tube; 2 per filter)

1. Wear gloves at all times.
2. Remove frozen filters from -80C.
3. Soak razor blades in 95% EtOH. You will need one sterile razor blade per filter.
4. Flame forceps. Remove two razor blades from EtOH. Allow to flame until EtOH is dry. CAUTION: Flaming EtOH will splatter from inside of the razorblades and will land on the bench. Take care not to set entire EtOH bath on fire.
5. Quickly place two sterile razorblades inside 1 sterile plastic Petri dish.
6. Prepare 2 beadbeater tubes per replicate. Each beadbeater tube should be sterile and contain 1 ceramic bead (sand should be removed if tube has come from a kit). Label tubes with sample name and replicate number,
7. Each filter will be divided in half between two beadbeater tubes. This division makes for optimal DNA extraction because it maximize cell lyses and minimize DNA shearing.
8. The top and the bottom of the Petri dish will each be used separately as a "cutting board" for the filer. Wash forceps in EtOH and flame. Sepatate Petri dish and place one razor blade on each half.
9. After thawing briefly, remove folded filter from cryulevial. Using aseptic techniques, and taking care to minimize handling of filter, slice the folded filter in half. Further slice filter halves into smaller squares.
10. Place half of each filter into a separate beadbeater tube. There should be two tubes per one filter.
11. Filters can now be re-frozen at -80C until DNA extraction.
12. Wash gloves with EtOH between cutting each filter.

IV. DNA Extraction

Needed: FastDNA extraction kit from Bio101 Systems
2 mL sterile microcentrifuge tube (2 per 1 filter; 1 per beadbeating tube)
Bead beater
Microcentrifuge

1. Wear gloves at all times. Remove frozen, cut filters from -80.
2. Add 1000 ul CLS-TC reagent to chopped filter in 2 mL beadbeating tube.
3. Beat at 3.5 for 30 seconds in bead beater (minibeadbeater-Biospec products).
4. Centrifuge tubes for 10 minutes at 13.0 rpm
5. Mix Binding Matriz Reagent vigorously. Pipette 600 uL of Binding Matrix reagent into 2 mL sterile tube. Add 600 uL of supernatant from beadbeater tube to Binding Matrix.
6. Incubate Binding Matrix and supernatant mixture for 5 min at room temperature.
7. Spin 2 mL tubes for 2 minutes.
8. Discard most of the supernatant; allow only enough supernatant to re-suspend precipitate. Use a pipetteman to resuspend precipitate, and add this suspension to a clean spin filter tube. *Pipette the precipitate from both filter halves into ONE spin filter tube. This step is a re-combining step.
9. Centrifuge spin filter tubes for one minute at 13.0 rpm. Discard flow-through (can be washed down sink).

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10. Wash spin filter with 500 uL SEWS solution (Note: a fresh bottle of SEWS from a new kit must be eluted with 100mL of 100% EtOH before use). Centrifuge for 1 minute.
11. Discard flow through. Wash again with 500 uL SEWS and centrifuge 1 minute. Discard flow-through.
12. Centrifuge 2 minutes additional minute to dry.
13. Remove spin filter from tube and place into sterile catch tube.
14. Elute with 100 uL DES (or sterile DI water).
15. Spin one minute. Save flow-through. Dispose of filter.
16. DNA is now PCR application ready. Store at -80C until Amplification.
17. Agarose Gel check: to ensure that the extraction was successful, check 5uL of product on 0.7% agarose gel. Use HindIII or 1000 bp ladder to determine molecular weight of bands (you will get a smear because we have extracted ALL DNA of different lengths from the sample), and use EtBr to visualize relative amount of DNA present.

IV. DNA quantification using NanoDrop at the Biotech Center

1. Bring gloves and 0-10 uL pipette tips
2. Nanodrop is located in the Genetics/biotech center. Enter from University Avenue entrance. Take elevator to 3rd floor. Turn left. Enter first door immediately on the left labeled "Gene Expression Center." Nanodrop is down the hall immediately inside the last entrance on the right.
3. Wash the nanodrop arm before using. Pipette 1-5 uL of water (provided) on to sensor, and close arm. Do not touch the Nanodrop by the cable- move only with the lever.
4. Open ND1000 software. Switch nucleic acid option (upper right of screen) to appropriate choice. DNA 50 is appropriate for Bio101 DNA extraction.
5. Initialize Nanodrop by pipetting a volume of buffer equal to the volume of sample that you will be measuring. The buffer should match your sample. Measure as blank.
6. Wipe Nanodrop arm and sensor with kimwipe.
7. Add a sample ID into the right box.
8. Add 1.5-5 uL of sample to the Nanodrop. Measure. Log will begin to fill automatically. You may check precision by measuring twice.
9. Wipe Nanodrop arm with kimwipe. It is unnecessary to wash with water between samples. Repeat steps 6-9 until all samples are quantified.
10. When finished with all samples, go to "Reports." Save report as text file in appropriate folder. E-mail yourself to import file into excel.
11. Before leaving, wash Nanodrop arm with 5 uL of water. Fill out log to the right of the machine with the date, type of buffer used, and type of nucleic acid measured.

V. DNA Amplication

Needed: PCR tube/ 96 well plate/strip tube
Thermocycler

Ingredient	per 1 PCR reaction (uL)
Sterile dH ₂ O	19-n
10x Idaho Tech Buffer	2.5
dNTPs (5 mM)	1.25

1406F* FAM-labeled (10 uM)	1.00
23Sr (10 uM)	1.00
Taq polymerase (Promega storage buffer B 18503717-M166A)	0.25
tDNA from DNA extraction	<i>n</i> (conc= 5ng/uL)
TOTAL	25 uL

Table 2. RISA PCR reagents.

1. Wear gloves for all steps. Also, use barrier micropipette tips.
2. Remove PCR reagents from freezer (-20C or -80C). Make appropriate mix (Table 1) for the amount of PCR reactions needed, except tDNA. If using a 96 well plate, use a trough and multi-channel pipettman to aliquot. Add Taq polymerase enzyme last, and mix PCR reaction thoroughly in tube.
3. From your quantification calculations, add a volume of your DNA extraction sample that has 5- 10 ng of tDNA.
4. Place tubes into thermocycler. Use the following PCR conditions for amplification:

Step	Temperature (C)	Time	Repeat
Denaturing	94	2 min	
Denaturing	94	35 sec	Repeat 30x total
Annealing	55	45 sec	
Elongation	72	2 min	
Elongation	72	2 min	

Table 3. RISA PCR thermocycler conditions.

5. PCR product can be stored at -20C until ARISA.
6. Agarose Gel check: to ensure that the amplification was successful, check 5uL of product on 0.7% agarose gel. Use HindIII ladder to determine mw of bands (You will get a smear because we have extracted ITS fragments of varying lengths. However, this smear should run between approximately 200 and 1750 bp. Use EtBr to visualize relative amount of DNA present.

VI. ARISA

Needed: strip tubes/96well plate
 10 uL formamide per 1 sample
 0.4 uL ROX standard per 1 sample (Bioventures- 2500 GeneScan)*
 *includes fragments: 100, 150, 200, 250, 300, 350, 400, 450, **475**,
 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1250,
 1500, 1750, 2000
 1 uL 1:1 diluted PCR product in ddH₂O.

1. Remove formamide from the freezer.
2. Dilute PCR product 1:1 with sterile ddH₂O. Remember, if you removed 5 uL of your product to run an agarose gel check, you must only dilute in 20 uL.
3. Mix the appropriate amount of formamide and ROX stand for the number of samples that you are running. They will be mixed in in a tube or trough, depending on how many samples you have. Aliquot 10 uL of the mixture into wells of a new plate/strip tube.

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4. Add diluted PCR product last. Seal (foil for a 96 well plate) and submit to Biotech Center. Service: RTG-GeneScan. A whole plate is costs 50 dollars. Specify that a long run to 2000 bp is needed. Optional: you can submit an excel spreadsheet with your sample names to the Biotech center for them to use in their analysis. This makes later sample sorting easier. <http://www.biotech.wisc.edu>

VI. ARISA fragment analysis : THIS ANALYSIS IS CONTINUALLY BEING IMPROVED. PLEASE INQUIRE AS TO THE LATEST PROTOCOL.

VII. Statistics

- A. PRIMER
- B. Correspondence Analysis/CANOCO
- C. vegan R package

Misc.

Primers:

1406F *6-FAM:

5'-TGYACACACCGCCCGT-3'

1 umol

no purifications

5' FAM label

23Sr

5' -GGGTTBCCCCATTCRG-3',

50 nmol desalted