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Biofilm DNA metabarcoding protocol

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We use this protocol and it's working

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Abstract

Full protocol for the extraction of DNA from river biofilm samples, 2-step PCR amplification of 16S rRNA, 18S rRNA, ITS2 and rbcL gene regions and amplicon sequencing.

Materials

Suggested kits, reagents and consumables

General consumables

- Starlab TipOne sterile filter tips, 1000 µl, cat# S1122-1730-C
- Starlab TipOne sterile filter tips, 200 µl, cat# S1120-8710-C
- Starlab TipOne sterile filter tips, 10/20 µl, cat# S1120-3710-C
- Starlab StarTub sterile reagent reservoirs, 55 ml, cat# E2310-1010
- Eppendorf DNA LoBind tubes, 1.5 or 2 ml, cat# 0030108078
- Sterile centrifuge tubes, 15 ml

DNA extraction

- Zymo Research Quick-DNA Fecal/Soil Microbe 96 Kit, cat# D6011
- Zymo Research DNA/RNA Shield, cat# R1100-250
- Proteinase K recombinant PCR grade
- Beta mercaptoethanol

DNA quantification

- Invitrogen Qubit dsDNA high-sensitivity quantification kit (single tube quantification), cat# Q32854
- Invitrogen Quant-iT dsDNA high-sensitivity quantification kit (plate quantification), cat# Q33120 or Promega QuantiFluor ONE dsDNA system, cat# E4870
- Invitrogen Qubit tubes, cat# Q32856
- Thermo Scientific Nunc F96 FluoroPlate black with lid, cat# 137101

PCR

- NEB Q5 high fidelity DNA polymerase, cat# M0491L
- Bioline dNTP mix 10 mM, cat# BIO-39053
- Molecular grade water
- Custom primers e.g. Integrated DNA Technologies
- Azenta Life Sciences FrameStar 96-well skirted low profile PCR plates, cat# PCR1220
- Thermo Scientific adhesive PCR plate seals, cat# AB0558

Gel electrophoresis

- Biotium GelRed, cat# 41003 or Invitrogen SybrSafe, cat# S33102
- Bioline HyperLadder 100 bp, cat# BIO-33029 or 1 Kb, cat# BIO-33025
- Agarose
- 10X TBE buffer

PCR clean up

- Millipore MultiScreen PCR 96-well plate, cat# LSKMPCR
- Qiagen TE buffer, cat# 19086

Normalisation

- Norgen NGS 96-well normalisation kit, cat# SKU61900
- Ethanol

Gel extraction

- Qiagen MinElute gel extraction kit, cat# 28604
- Isopropanol

1. DNA extraction

1h 25m

1 Defrost and gently vortex samples to resuspend sample material.

> **Note**
>
> DNA will be extracted using the Zymo Research Quick-DNA fecal/soil microbe 96 kit following an amended version of the manufacturer's protocol to maximise DNA yield. Use sterile filter pipette tips throughout.

2 Transfer 100 µL of sample material to each tube of the 96-tube lysis rack. Make a note of sample positions and leave at least one tube free of sample material as a negative extraction control.

3 Add 500 µL of Zymo DNA/RNA shield to each tube of the lysis rack and seal rack with provided tube cap films.

4 Secure in a bead beater such as the Qiagen TissueLyser II and lyse at 20 Hz for 00:20:00. 20m

5 Before uncapping the tube rack, centrifuge at 3000 x g for 00:05:00. 5m

6 Add 20 µL of Proteinase K to each tube of the lysis rack.

7 Incubate at 65 °C for 00:20:00. 20m

8 Before uncapping the tube rack, centrifuge at 3000 x g for 00:05:00. 5m

> **Note**
>
> The following steps are as described in the manufacturers protocol.

9 Transfer 250 µL of lysed supernatant from the lysis rack to the same position in a 96-well block.

10 Add 750 µL beta-mercaptoethanol (BME) to the genomic lysis buffer and invert the bottle to mix.

Safety information

Always work in a fume hood when working with beta-mercaptoethanol (steps 10-18).

11 Add 750 µL of genomic lysis buffer with BME added to each well of the 96-well block, pipette up and down to mix. Seal with an adhesive plate seal.

12 Centrifuge at 3000 x g for 00:05:00 . 5m

13 Remove the plate seal and transfer 500 µL from each well of the 96-well block to the same position in a silicon-A plate mounted on a collection plate. Seal the silicon-A plate with an adhesive plate seal.

14 Centrifuge at 3000 x g for 00:05:00 . Discard the flow through from the collection plate into a hazardous waste bottle and return the silicon-A plate to the same collection plate. go to step #13 until all supernatant has been processed. 5m

15 Remove the plate seal and add 200 µL of DNA pre-wash buffer to each well of the silicon-A plate mounted on the emptied collection plate. Replace the adhesive plate seal.

16 Centrifuge at 3000 x g for 00:05:00 . Discard the flow through from the collection plate into a hazardous waste bottle and return the silicon-A plate to the same collection plate. 5m

17 Remove the plate seal and add 500 µL of g-DNA wash buffer to each well of the silicon-A plate mounted on the emptied collection plate. Replace the adhesive plate seal.

18 Centrifuge at 3000 x g for 00:05:00 . Discard the flow through from the collection plate into a hazardous waste bottle. 5m

Note

Place the silicon-A plate aside until step 25. The following steps can now be performed outside of the fume hood.

19 Place a HRC plate on an elution plate and pierce the cover foil. Add 150 µL of prep solution to each well of the HRC plate and leave at room temperature for 00:05:00. 5m

20 Centrifuge at 3000 x g for 00:05:00. Discard the flow through from the elution plate into a hazardous waste bottle. 5m

21 Remove the plate seal from the silicon-A plate and place onto the prepared HRC plate, place the assembly onto a new elution plate to form a 3-plate stack. Add 100 µL of DNA elution buffer to the silicon-A plate and replace the adhesive plate seal.

22 Centrifuge at 3500 x g for 00:05:00. 5m

23 Seal the elution plate with an adhesive plate seal, label and store at 4 °C in a fridge short-term (up to 1 week) or in a freezer at -20 °C to -70 °C for long term storage.

2. DNA quantification 10m

24 To quantify using Nanodrop, clean each pedestal of the Nanodrop spectrophotometer with molecular grade water and blank the instrument with 1 µL of elution buffer as instructed by the Nanodrop software. Transfer 1 µL of each DNA sample to a pedestal, shut the lid and click measure. Export the results or make a note of the DNA concentration and the 260/280 and 260/230 ratios.

25 To quantify using the QuantiFluor ONE dsDNA kit, prepare DNA standards in Eppendorf tubes as shown in Table 1. 5m

	Standard	Volume of standard (µL)	Volume of TE buffer (µL)	Concentration (ng/µL)
	A	15 of Lambda DNA	0	400
	B	10 of A	10	200
	C	5 of B	15	50
	D	5 of C	15	12.5
	E	5 of D	15	3.1
	F	5 of E	15	0.8
	G	5 of F	15	0.2

Table 1. Preparation of DNA standard dilutions.

Add 200 µL of QuantiFluor dye to each well of a black microplate and add 1 µL of each standard dilution in duplicate or triplicate, 1 µL TE buffer as a negative control, or

1 µL of DNA. Gently pipette up and down or vortex to mix and incubate the plate at room temperature for 00:05:00. Place the plate in a spectrophotometer such as the BioTek Cytation 5 imaging reader and measure fluorescence at 504 nm excitation and 531 nm emmision. Calculate the DNA concentration according to the standard curve.

26 To quantify using the Quant-iT dsDNA high-sensitivity kit, dilute the reagent 1:200 in buffer in a plastic centrifuge tube and invert to mix. Add 200 µL of this solution to each well of a black microplate. Add 10 µL of the provided standards or 10 µL of DNA to the wells. Gently pipette up and down or vortex to mix and incubate the plate at room temperature for 00:05:00. Place the plate in a spectrophotometer such as the BioTek Cytation 5 imaging reader and measure fluorescence at 502nm excitation and 523 nm emmision. Calculate the DNA concentration according to the standard curve. 5m

3. Step 1 PCR

27 Order primers with the Illumina adaptor sequences attached at the 5' end according to Table 2 and 3.

	Target gene	F primer	F primer sequence (5'-3')	R primer	R primer sequence (5'-3')	Reference
	16S rRNA	515f	GTGYCAGCMGCCGCGGTAA	806r	GGACTACNVGGGTWTCTAAT	Walters et al. (2016)
	18S rRNA	NSF573	CGCGGTAATTCCAGCTCCA	NSR951	TTGGYRAATGCTTTCGC	Mangot et al. (2012)
	ITS2	ITS7F	GTGARTCATCGAATCTTTG	ITS4R	TCCTCCGCTTATTGATATGC	Ihrmark et al. (2012)
	rbcL	rbcL-646F	ATGCGTTGGAGAGARCGTTTC	rbcL-998R	GATCACCTTCTAATTTACCWACAACTG	Kelly et al. (2020)

Table 2. Amplicon forward (F) and reverse (R) sequences for 16S rRNA (bacteria), 18S rRNA (eukaryotes), ITS2 (fungi and other eukaryotes) and rbcL (diatoms and other phototrophs) gene regions.

	Adaptor direction	Sequence (5'-3')
	Forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
	Reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

Table 3. Illumina adaptor sequences.

28 Defrost PCR reagents in Table 2 and gently vortex to mix before use. Clean the laminar flow hood with ethanol and then place all pipettes, pipette tips, reagent reservoirs, centrifuge tubes, plates, plate seals and gloves in a laminar flow hood for UV-sterilisation.

29 In the flow hood, combine the PCR reagents in the order presented in a centrifuge tube to form a master mix for the number of samples required according to Table 4. Add 5% to the number of samples the master mix is prepared for to account for pipetting error or loss. Gently invert to mix.

Reagent	Volume per sample (µL)
Molecular grade water	26.3
5X reaction buffer	10
5X high GC enhancer	10
dNTPs (10 mM)	1
Forward primer (100 µM)	0.1
Reverse primer (100 µM)	0.1
Q5 taq (2000 units/ml)	0.5

Table 4. Volume of PCR reagents per sample for 50 µL reactions.

30 Dispense the master mix into a reagent reservoir and transfer 48 µL to each well of a PCR plate.

31 Add 2 µL of DNA to each well and make a note of sample positions. Pipette up and down to mix. leave at least one well free of DNA as a negative PCR control, or add 2 µL of negative extraction blank. Seal with an adhesive plate seal.

32 Centrifuge at 1000 x g for 00:01:00 . 1m

33 Place the plate in a thermocycler and run the relevant program for each primer in Table 5.

Target gene	Stage	Temperature (°C)	Duration	No. cycles
16S rRNA	Initial denaturation	95	2 min	
	Denaturation	95	15 sec	30
	Annealing	50	30 sec	
	Extension	72	30 sec	
	Final extension	72	10 min	
18S rRNA	Initial denaturation	94	5 min	
	Denaturation	94	30 sec	30

	Target gene	Stage	Temperature (°C)	Duration	No. cycles
		Annealing	60	30 sec	
		Extension	72	30 sec	
		Final extension	72	10 min	
	ITS2	Initial denaturation	95	3 min	
		Denaturation	95	15 sec	30
		Annealing	52	30 sec	
		Extension	72	30 sec	
		Final extension	72	10 min	
	rbcL	Initial denaturation	98	2 min	
		Denaturation	98	20 sec	35
		Annealing	55	45 sec	
		Extension	72	1 min	
		Final extension	72	5 min	

Table 5. PCR thermocycling conditions for each amplicon primer. Denaturation, annealing and extension stages are repeated for X number of cycles.

34 Prepare a [M] 1.5 % (v/v) agarose gel to visualise PCR product. For example, for a maxi gel (up to 4 combs of 52 wells), combine 250 mL of 1X TBE buffer with 3.75 g agarose in a conical flask. Heat in a microwave until all of the agarose has dissolved, allow to cool to approximately 50 °C and add 5 mL GelRed or SybrSafe DNA stain. Mix well and pour into the gel tray with combs in place and secured in a gel caster. Allow to set for approximately 00:20:00. Once set, carefully remove the combs and transfer the gel within the gel tray to a gel tank and submerge in 1X TBE buffer. 20m

35 Transfer 5 µL of PCR product to a microplate and add 1 µL of loading buffer to each well. Transfer each sample to the wells of the gel. Add 5 µL of 100 Kb hyperladder to at least one well per row and run the gel at 130 V for 00:45:00. 45m

36 Carefully place the gel on a gel imager such as the BioRad GelDoc imager to visualise the gel and determine if PCR amplification was successful and the PCR product is of the expected size according to the ladder.

4. PCR clean up

37 Transfer 20 µL of PCR product to a Millipore MultiScreen PCR plate and add 80 µL of TE buffer, pipette up and down to mix and avoid bubbles.

38 Place the plate onto a vacuum manifold and apply vacuum at 0.7 Bar for 00:12:00, making sure the wells are completely empty before removing from the vacuum. Blot droplets. 12m

39 Add 35 µL of molecular grade water to each well and vortex at 1100 rpm for 00:10:00. 10m

40 Transfer cleaned PCR product to a PCR plate and store at 4 °C in a fridge short-term (up to 1 week) or in a freezer at -20 °C for long term storage.

5. Step 2 PCR

41 Defrost and centrifuge primers at 1000 x g for 00:01:00. Prepare primer array plates for dual-indexing of samples by using a liquid handling robot such as Opentrons to transfer 20 µL of each forward and reverse indexing primer pair at 50 micromolar (µM) to 360 µL of molecular grade water in a 96-deep well block for a final primer concentration of 0.5 micromolar (µM). Transfer an aliquot of 5 µL from the master array plate to a separate plate for PCR, respecting plate positions. Seal all plates with an adhesive plate seal. 1m

Note

Second step PCR was performed using a dual-indexing approach. This barcoded design allows sequences to be fully demultiplexed into individual samples with the use of a series of forward and reverse index sequences combined in unique primer pairings (Kozich et al., 2013). Each indexing primer consisted of a forward (i5) or reverse (i7) Illumina adaptor sequence (forward adaptor: 5'-AATGATACGGCGACCACCGAGATCTACAC-3', reverse adaptor: 5'-CAAGCAGAAGACGGCATACGAGAT-3'), an 8 nucleotide i5 or i7 Nextera index sequence and an Illumina pre-adaptor sequence (forward pre-adaptor: 5'-TCGTCGGCAGCGTC-3', reverse pre-adaptor: 5'-GTCTCGTGGGCTCGG-3'). i5 and i7 index sequences can be found at https://mothur.s3.us-east-2.amazonaws.com/wiki/wet-lab_miseq_sop.pdf.

42 Defrost PCR reagents in Table 6 and gently vortex to mix before use. Defrost the cleaned step 1 PCR product and the aliquoted primer array plate and centrifuge at 1000 x g for 00:01:00. Clean the laminar flow hood with ethanol and then place all pipettes, pipette tips, reagent reservoirs, centrifuge tubes, plate seals and gloves in a laminar flow hood for UV-sterilisation. 1m

43 In the flow hood, combine the PCR reagents in the order presented in a centrifuge tube to form a master mix for the number of samples required according to Table 6. Add 5% to the number of samples the master mix is prepared for to account for pipetting error or loss. Gently invert to mix.

Reagent	Volume per sample (µL)
Molecular grade water	7.25
5X reaction buffer	5
5X high GC enhancer	5
dNTPs (10 mM)	0.5
Q5 taq (2000 units/ml)	0.25

Table 6. Volume of PCR reagents per sample for 25 µL reactions. Denaturation, annealing and extension stages are repeated for 8 cycles.

44 Dispense the master mix into a reagent reservoir and transfer 18 µL to each well of the aliquoted primer array plate, taking care not to touch primers in the plate with the pipette tip.

45 Add 2 µL of cleaned step 1 PCR product to each well and make a note of sample positions. Pipette up and down to mix. leave at least one well free of DNA as a negative PCR control, or add 2 µL of negative step 1 PCR blank. Seal with an adhesive plate seal.

46 Centrifuge at 1000 x g for 00:01:00 . 1m

47 Place the plate in a thermocycler and run the program in table 7.

Stage	Temperature (°C)	Duration	No. cycles
Initial denaturation	95	2 min	
Denaturation	95	15 sec	8
Annealing	50	30 sec	
Extension	72	30 sec	
Final extension	72	10 min	

Table 7. PCR step 2 thermocycling conditions

48 go to step #34 for gel electrophoresis.

49 Store at 4 °C in a fridge short-term (up to 1 week) or in a freezer at -20 °C for long term storage.

6. Normalisation

50 Use the Norgen NGS normalisation kit to normalise DNA to 5 ng/µL. Add 60 µL of buffer SK to 20 µL of PCR product in the PCR plate, pipette up and down to mix and then transfer all to the 96-well normalisation plate mounted on top of a collection plate. Seal with an adhesive plate seal.

51 Centrifuge at 3200 x g for 00:02:00. Discard the flow through from the collection plate into a hazardous waste bottle. 2m

52 Add 90 mL 100% ethanol to wash solution A, invert the bottle to mix. Remove the plate seal and add 400 µL of wash solution A with ethanol added to each well of the normalisation plate on the emptied collection plate. Replace plate seal.

53 Centrifuge at 3200 x g for 00:02:00. Discard the flow through from the collection plate into a hazardous waste bottle. 2m

54 go to step #52 to repeat the wash step. After the second wash step, centrifuge at 3200 x g for 00:15:00. 15m

55 Place the normalisation plate on top of an elution plate, remove the plate seal and add 100 µL of elution buffer to each well.

56 Centrifuge at 200 x g for 00:01:00 and then at 3200 x g for 00:05:00. 6m

57 Pool normalised PCR product by plate into a centrifuge tube.

58 Quantify the plate pool with the Qubit dsDNA high sensitivity kit. Dilute stain 1:200 in buffer in a centrifuge tube. Add 190 µL of solution to a Qubit tube and add 10 µL sample or standard (scale for the number of samples required, including 2 standards and add 2 for 2m

pipetting error or loss). Vortex to mix. Incubate at room temperature for 00:02:00 before measuring the DNA concentration of the standards and then of the samples on the Qubit fluorometer.

59 For all of the plate pools that will be sequenced on the same sequencing run, dilute an aliquot of each plate pool with elution buffer to achieve a normalised concentration in 50 µL.
Pool all of the plate pools of each amplicon (taking into account sample numbers if each plate had a different number of samples) to form the normalised library (pool different amplicons separately).

7. Gel extraction 1h 29m

60 Vacuum concentrate the library to approximately 30 ul.

61 Prepare a [M] 2 % (v/v) agarose gel. For example, for a mini gel (up to 1 combs of 8 wells), combine 100 mL of 1X TBE buffer with 2 g agarose in a conical flask. Heat in a microwave until all of the agarose has dissolved, allow to cool to approximately 50 °C and add 5 mL GelRed or SybrSafe DNA stain. Mix well and pour into the gel tray with combs in place and secured in a gel caster. Allow to set for approximately 00:10:00. Once set, carefully remove the combs and transfer the gel within the gel tray to a gel tank and submerge in 1X TBE buffer. 10m

62 Add 10 µL of loading buffer to the library and transfer to a well in the gel. Add 5 µL of 100 Kb hyperladder to at least one well and run the gel at 90 V for 01:00:00. 1h

63 Place the gel on a UV light box and with a sterile scalpel, cut the library band from the gel and place in a pre-weighed Eppendorf tube. Weigh the Eppendorf after adding the gel slice to calculate the weight of the gel slice.

64 Use the Qiagen gel extraction MinElute kit to extract the purified library from the gel slice. Add 3 volumes of buffer QG to 1 volume of gel (100 mg gel = 100 µL) in the Eppendorf tube.

65 Incubate at 50 °C for 00:10:00 and invert the tube periodically. Make sure the gel has fully dissolved. 10m

66 Add 1 volume of isopropanol to the sample and invert to mix.

67 Transfer up to 700 µL of the sample to a MinElute spin column in a collection tube and centrifuge at 17900 x g for 00:01:00 . Discard the flow through into a hazardous waste bottle. Repeat until all of the sample has been filtered. 1m

68 Add 100% ethanol to buffer PE according to the label on the bottle. Add 750 µL buffer PE with ethanol added to the spin column in an emptied collection tube. Let the spin column stand for 00:05:00 . 5m

69 Centrifuge at 17900 x g for 00:01:00 . Discard the flow through into a hazardous waste bottle and repeat the centrifugation step. 1m

70 Place the spin column into an Eppendorf tube and add 100 µL of elution buffer. Let the spin column stand for 00:01:00 . 1m

71 Centrifuge at 17900 x g for 00:01:00 . 1m

72 go to step #58 to quantify the gel extracted library. Aim for a concentration of 0.2-0.6 ng/ul and dilute with elution buffer if necessary.

8. Library preparation and sequencing

73 Calculate the molarity (nM) of each amplicon according to its length in bp and calculate the concentration of the library in nM using the Qubit reading. If multiple amplicons are to be sequenced on the same run, pool in an equimolar ratio (taking into account number of samples and amplicon molarity). Dilute to a final loading library concentration of [M] 1000 picomolar (pM) if sequencing on the Illumina NextSeq platform with onboard denaturation, or to [M] 400 picomolar (pM) if sequencing on the Illumina MiSeq platform with manual denaturation.

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