



May 09, 2022

18S-V4 rRNA amplification from sorted cells (low-concentration DNA) for NGS Illumina sequencing (Metabarcoding)

Estelle Bigeard¹, Adriana Lopes Dos Santos², Catherine Ribeiro³¹AD2M, Station Biologique de Roscoff, CNRS, SU; ²Asian School of Environment - NTU;³Station Biologique de Roscoff

1

dx.doi.org/10.17504/protocols.io.81wgb71k1vpk/v1

Ecology of Marine Plankton (ECOMAP) team - Roscoff

Roscoff Culture Collection

1



Estelle Bigeard

AD2M, Station Biologique de Roscoff, CNRS, SU

For metabarcoding purpose, the first step involves the amplification by PCR of a given gene region (for example V4 or V9 region of 18S rRNA gene) or gene itself if its size does not exceed 600bp (the highest fragment size that can be sequenced by Illumina technology).

The defined forward and reverse primers that are complementary upstream and downstream of the region of interest, needs to be designed with overhang adapters which will be used in a subsequent limited-cycle amplification step, in order to add the dual-index barcodes and Illumina flow cell adapters. To design illumina primers, it will be necessary to know the sequencing method, and therefore the adapters sequence.

The following protocol explains the generation of 18S-V4 PCR amplicons for eukaryote metabarcoding analyses on sorted cells from seawater samples. Here we described the amplification protocol for 2 sequencing platforms (Genomer, Roscoff, France & Get-PlaGE, Toulouse, France).

DOI

dx.doi.org/10.17504/protocols.io.81wgb71k1vpk/v1

Estelle Bigeard, Adriana Lopes Dos Santos, Catherine Ribeiro 2022. 18S-V4 rRNA amplification from sorted cells (low-concentration DNA) for NGS Illumina sequencing (Metabarcoding). **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.81wgb71k1vpk/v1>



S. Metz et al - Has been uploaded to ScholarOne. Diversity of photosynthetic picoeukaryotes in eutrophic shallow lakes as assessed by combining flow cytometry cell-sorting and high throughput sequencing - FEMS Microbiology Ecology, 2019, DOI: 10.1093/femsec/fiz038

S. Metz et al. Freshwater microeukaryotes: unveiling the unexplored in a large floodplain system. Environmental Microbiology , 2021, hal-03611307, doi.org/10.1111/1462-2920.15838

gene amplification, metabarcoding, NGS, sequencing, eukaryote, sorted cells

protocol ,

Sep 23, 2020

May 09, 2022

42409

Lepère, C. et al. Whole Genome Amplification (WGA) of marine photosynthetic eukaryote populations. FEMS Microbiol. Ecol. 76, 516–523 (2011).

Stoeck, T. et al. Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. Molecular Ecology, 19 (Suppl. 1), 21–31 doi: 10.1111/j.1365-294X.2009.04480.x (2010).

Piredda, R. et al. Diversity and temporal patterns of planktonic protist assemblages at a Mediterranean Long Term Ecological Research site. FEMS Microbiology Ecology 93:fiw200. DOI: 10.1093/femsec/fiw200 (2017).

• Equipments

PCR hood (UV)
Thermocycler
Spark & Nanoquant Plate (Tecan)
Qubit 4 fluorometer (Invitrogen)
Microwave
Electrophoresis system & generator (BioRad)
Image Quant LAZ4000 (Ge Healthcare)

• PCR amplification

Primers Quality SePOP in water, stock 100µM - Eurogentec
Primers with Illumina tail Quality NGS Purification RP-HPLC, dried - Eurogentec

Nuclease-Free Water - Invitrogen™ Ambion™ - Ref AM9937
Phusion® High-Fidelity PCR Master Mix with HF Buffer - New England Biolabs - Ref M0531S
BSA - New England Biolabs - Ref B9000S
KAPA HiFi HotStart ReadyMix® - Roche Diagnostic France - Ref KK2602

nuclease free 96-wells PCR plates
1.5ml microtubes

• Electrophoresis

50X TAE Buffer :

- *Tris-Base (2M final) 242.5 g*
- *Acetic acid (>99.7%, 2M final) 7 ml*
- *0,5 M EDTA pH 8,0 (50 mM final) 100 ml*

dH2O up to 1L

Tris-Base - Sigma Aldrich - Ref T6791
Acetic acid - Thermo Fisher Scientific - Ref 11463473
0,5 M EDTA pH 8,0 - Fisher Scientific - Ref 15575-020
agarose - Interchim - Ref 31272L
Ethidium bromide - Sigma Aldrich - Ref E1510
SmartLadder - 200 to 10000 bp - Eurogentec - Ref MW-1700-10

• Divers

Tris - Thermo-fisher - Ref AM9855G
EDTA - Thermo-fisher - Ref15575020
Triton x-100 - Sigma Aldrich -ref T8787

Samples : Acid nucleic extraction and quantification

- 1 Extract DNA/RNA from the samples using the method/kit appropriate to the specific samples.

Filtered environmental samples usually have more DNA than sorted cells samples, so the V4 region of the 18S rRNA gene can be amplified by a single PCR reaction (without the need of a

nested protocol).

Sorted populations by flow cytometer can be used as an input for the PCR reactions. Photosynthetic pico and nanoeukaryotes populations selected based on light scatter, orange phycoerythrin and red chlorophyll fluorescence are sorted in sterile eppendorf tubes containing Tris-EDTA lysis buffer (Tris 10 mM, EDTA 1 mM and Triton x-100 1.2%). The Tris, EDTA and water use should be molecular grade. A buffer containing Tris-HCl 10 mM, pH 8.0 and NaCl 20 mM and filtered at 0.22 μ m should be used as sheath liquid for marine samples. Sheath fluid samples should be collected and analyzed as negative controls in all subsequent steps including sequencing, in order to test for contamination in the flow sorting process. After the sorting, samples can be stored at -80C. The DNA from sorted cells can be extracted by three cycles of freezing in liquid nitrogen and thawing in water.

When working with RNA, another step to transform the RNA in cDNA is necessary.

Quantify and quality-check the final DNA via NanoDrop or Qubit/PicoGreen Method.

The A280/260 ratios should be 1.8 or higher and once working with total community extracted DNAs, a concentration of at least 1 ng/ μ L is desirable to get consistent PCR results. Avoid secondary extractions or clean-ups for inhibitors, until PCRs have truly shown inhibition (example after trying the PCR by diluting the original DNA and/or adding BSA to the final PCR reaction) – many suboptimal DNAs will still work in PCR. A gel can be run to verify integrity of the extracted material, but it is generally unnecessary for PCR-only studies (**Figure 1**). Some protocols suggest the normalization of the DNA samples concentration prior to the PCR. In our experience, this is not always possible given the low concentration of some samples. The volume of sample added to the PCR can be settled in function of a range of concentrations instead of normalizing all the samples to the same concentration.

Load in agarose gel 0.8%, 1 μ L DNA + loading buffer. The limit of DNA detection using EtBr gels is around 10ng.

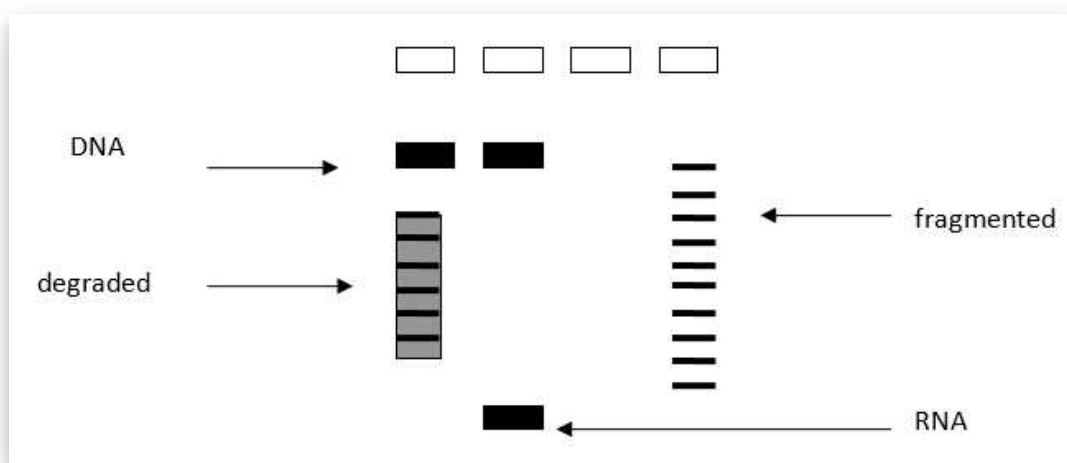


Figure 1: DNA/RNA detection in agarose gel.

Each DNA sample have to be normalized to 5ng/ μ l for PCR. For that, dilute DNA in nuclease free water.

It is not necessary to check the DNA concentration after dilution.

Samples with a DNA concentration less than 5ng/μl will be amplified as is in PCR.

PCR amplification

- Below is a suggestion of protocols for 18S amplification from sorted cells. This protocol has been tested and used with success.

However, before starting the library preparation, few samples from the batch (es) to be analyzed should be selected and the PCR conditions should be tested. The tests can be performed by using the primers without the illumina “tail” which are less expensive since it can be order by normal purification method, like Se-POP. By using also these primers, it avoids excessive manipulation of the primers with the illumina tail and therefore the chances of contamination. Low number of cycles, pool multiple (i.e., triplicate) PCRs for each sample, high initial time of denaturation are fundamental practices for the PCR when targeting diversity studies and should be considered for all reactions, including the tests in order to reduce or avoid PCR bias caused by PCR selection and PCR drift.

- **PCR selection** includes all mechanisms which inherently favor the amplification of certain templates due to properties of the genes and/or of their flanking sequences of the target region. Potentially important contributors to PCR selection are preferential denaturation due to overall low GC content, higher binding efficiency of GC-rich permutations of degenerate primers, differential accessibility of rRNA genes within genomes, and correlation between amplification probabilities and gene copy numbers within genomes.

- **PCR drift** is caused by stochastic variation in the early cycles of the reaction (when amplification still proceeds largely from the genomic templates), and its outcome should therefore not be reproducible in replicate PCR amplifications.

More details about these biases can be found in the following references:

-Aird, D. et al. Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries. *Genome Biol.* 12, R18 (2011).

-Wagner, A. et al. Surveys of Gene Families Using Polymerase Chain Reaction: PCR Selection and PCR Drift. *Syst. Biol.* 43, 250 (1994).-Polz, M. F. & Cavanaugh, C. M. Bias in template-to-product ratios in multitemplate PCR. *Appl. Environ. Microbiol.* 64, 3724–3730 (1998).

Primers used for DNA amplification and sequencing

2.1

Use the existing primers (Table 1 & Table 2) or design your own custom gene primers with the proper Illumina indices and Index adaptor orientations. These adapters will be different in function of the libraries preparation protocol, and therefore in function of sequencing platform (Table 2).

PCR amplification of the V4 region of the 18S rDNA (~380 bp) using primers TAREuk454FWD1 (V4F) and V4 18S Next.Rev (TAREukREV3_modified or V4R_Zig) allow to amplify most eukaryotic groups and to solve the problem of the under-representation of Haptophytes.

Sorted cells samples usually have less DNA than filtered samples, so the V4 region of the 18S rRNA gene can be amplified by a nested PCR reaction.

Primers for the first round can be order by normal purification method, like Se-POP or RP-Carttridge.

It is recommended to order the illumina primers with a good synthesis and purity parameters.

We have ordered at EUROGENTEC the NGS primers with RP-HPLC purity method, 10 or 40nmol synthesis scale and at 100µM TE concentration. We could also order in dried and dissolve them with nuclease free water or TE 1x buffer to obtain 100µM stock solution (as described on the Eurogentec's technical data sheet).

Then we dilute them to 10 µM working concentration (1/10th the typical 100 µM working stock concentration for primers). This proceed should be performed at the PCR hood and with molecular grade water.

The primers (stock and working solutions) are stored at -20°C.

Primer	Product size	Sequence (5'-3')	Reference
V4F	406	CCA GCA SCY GCG GTA ATT CC	Stoeck et al. 2010
V4R_Zig		ACT TTC GTT CTT GAT YRA TGA	Stoeck et al. 2010
63F		ACGCTTGTCTCAAAGATTA	Lepère et al. 2011
1818R		ACGGAAACCTTGTTACGA	Lepère et al. 2011

Table 1: Primers used for DNA amplification and sequencing

Primer	Product size with the tail	Sequencing by:	Final Primer (Illumina tail)
V4F_illumina	527	Genomer (Roscoff)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCAGCASCYGCGGTAATTCC
V4R_Zig_illumina			GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACTTTCGTTCTTGATYRATGA
V4F_illumina	527	Genotoul	CTTCCCTACACGACGCTCTTCCGATCT CCAGCASCYGCGGTAATTCC
V4R_Zig_illumina			GGGAGTTCAGACGTGTGCTCTTCCGATCT ACTTTCGTTCTTGATYRATGA

Table 2: 18S final primers with Illumina tail

Controles

2.2 Test a negative control for each series. The negative PCR product could be

inserted in the library to have a control of air contaminations.

It is possible to test a positive control (mock communities) to check the sequencing efficiency and sequencing error. For that, you have to select axenic cultures, to know the starting cells concentration and the DNA concentration. We could buy commercial Mock communities (ex: Zymo).

With Kapa HotStart ReadyMix® (2 rounds)

2.3 a) First round

Place plates, tubes and nuclease free water to prepare the PCR mix under UV.

From sorted cells, the first round of 18s PCR amplification is done in triplicate, as follows:

Reagent	Initial concentration	Final concentration	Volume
Primer 63F	10 μ M	0.3 μ M	0.3 μ L
Primer 1818R	10 μ M	0.3 μ M	0.3 μ L
KAPA HiFi HotStart ReadyMix®	2x	1x	5 μ L
DNA			1 μ L
H2O up to a 10 μ L reaction			3.4 μ L

Table 3: PCR reaction using KAPA HiFi HotStart ReadyMix® for the first round

		Temperature (°C)	Time	25 cycles
Initial denaturation		95°C	5 min	
Amplification	Denaturation	98°C	20 sec	
	Annealing	52°C	30 sec	
	Elongation	72°C	90 sec	
Final elongation		72°C	5 min	

Table 4: Thermal conditions using KAPA HiFi HotStart ReadyMix® for the first round

a) Second round

The second round is performed with the same reaction conditions using the primers V4F_illumina and V4R_Zig_illumina and 1.0 μ L of the pooled 1st round product reaction as follows:

Reagent	Initial concentration	Final concentration	Volume
Primer V4F_illumina	10 μ M	0.3 μ M	0.6 μ L
Primer V4R_Zig_illumina	10 μ M	0.3 μ M	0.6 μ L
KAPA HiFi HotStart ReadyMix®	2x	1x	10 μ L
Pooled 1 st round product reaction			2 μ L
H2O up to a 20 μ L reaction			6.8 μ L

Table 5: PCR reaction using KAPA HiFi HotStart ReadyMix® for the second round

Thermal conditions of the second round are:

		Temperature (°C)	Time	25 cycles
Initial denaturation		95°C	3 min	
Amplification	Denaturation	98°C	20 sec	
	Annealing	65°C	1 min	
	Elongation	72°C	90 sec	
Final elongation		72°C	5 min	

Table 6: Thermal conditions using KAPA HiFi HotStart ReadyMix® for the second round

Gel electrophoresis

- PCR products should be checked initially by gel electrophoresis for unspecific amplification and band size.

- Check PCR products on agarose gel to see if the bands are weak or strong.

Prepare an agarose gel 1-1.5% in 0.5X TAE buffer

Microwave until the solution is clear (2 minutes)

Stain with 20 μ L ethidium bromide per 100ml (or SYBR-safe dye)



[Ethidium bromide 10 mg/ml Sigma](#)

Aldrich Catalog #E1510



Be careful : Put labcoat, nitriles gloves!

Prepare tray & comb(s)

Pour gel into tray

When the gel is polymerized, remove comb

Load gel in electrophoresis cuve containing the same 0.5X TAE buffer

Gel must be covered

Cut one strip of parafilm.

Load 1-2 µL loading dye onto parafilm, one drop per sample

Mix 1 to 3 µl of each PCR product with loading dye (up & down 3 times)

Load it in the agarose gel

Load 3 µL of SmartLadder into first and/or last well

Close & Plug in electrodes to power source

Run the migration for ~120 V, 30-45 minutes

Observe results using a UV imager like Image Quant LAZ4000, Ge Healthcare

The **negative control must be negative** and the **positive control must be positive** to validate the PCR.

The size of amplicons is indicated in Table 1 and 2.

- Samples with failed PCRs (or spurious bands) are re-amplified by optimizing the PCR (further template dilution to 1:100 or using BSA/other additives) to produce correct bands, or purified using a purification kit like "Nuclesopin Gel & Clean up kit– Macherey-Nagel".

Amplicons are stored at -20°C until treatment.

Preparation of libraries for Illumina sequencing (metabarcoding)

- 4 If the PCR's product are not concentrated enough, you could purifie and concentrate them in a smaller volume using a purification and concentration kit like "Nuclesopin Gel & Clean up kit– Macherey-Nagel".

Once PCR products are correct, they are pooled and organized in appropriated PCR plates to generate libraries.

We use a high throughput sequencing using paired-end 2x300bp Illumina MiSeq with the 600-cycle v3 kit.

If the illumina sequencing is realized on the Genomer Platform in Roscoff (France), see the protocols.io "Preparation of libraries (Metabarcoding) for Illumina sequencing - Genomer Platform".

If the sequencing is realized by another sequencing service, send to the service pictures of gel to check they are conform (purity, degradation, bands intensity, etc) and follow its protocol (quantification, purification etc).

