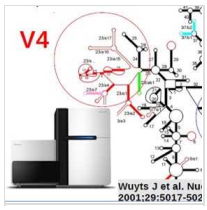

Feb 14, 2022

Eukaryotes 18S-V4 rRNA Metabarcoding PCR protocol for NGS Illumina sequencing

Sarah Romac[1]

[1]UMR7144-Roscoff Marine Station

dx.doi.org/10.17504/protocols.io.bzucp6sw

Ecology of Marine Plankton (ECOMAP) team - Roscoff AD2M

Sarah Romac
UMR7144-Roscoff Marine Station

Nowadays metabarcoding approaches allow to explore the diversity of different communities using next-generation sequencing (NGS).

Here we describe the 18S-V4 DNA amplification method applied for eukaryote metabarcoding analyses using Illumina Miseq technology. This protocol has been used in many projects studying eukaryotic diversity (TARA-OCEANS 2009-2013, TARA-PACIFIC 2016-2018), and eukaryote monitoring projects (MOOSE-GE 2017-..., ROSCOFF ASTAN 2009-ongoing).

We developed the flowchart for 2 different sequencing platforms : Fasteris-Gene Support SA (Plan-Les-Ouates, Swiss) and GeT-PlaGE (Toulouse, France).

DOI

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PCR, NGS, metabarcoding, eukaryotes, 18S-V4

protocol ,

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54884

As metabarcoding is very sensitive to contaminations by exogen DNA, please respect some conditions :
- always wear a labcoat, and clean nitrile gloves;
- separate the work area for prePCR and postPCR manipulations.
- do your PCR under a PCR hood.

Specific Equipment (more details in the concerned steps) :
- PCR hood equipped with UV light and HEPA filter ;
- Thermocycler.
- Qubit 4 Fluorometer (Invitrogen) ;
- Fluorometer Plate reader ;
- Gel Tray Caster and Imager .

Optional Equipment :
- 2100 Bioanalyzer Instrument (Agilent)

Supplies :
Sterile microtubes 1,5mL
Semi-skirted PCR plates 0.2mL, 96 wells (like AB-0900) and thermoresistant seals.
Filter tips

Reagents and kis are mentioned in the protocol in the concerned steps.

Do aliquots of 1 mL of sterile milliQ water.

Before starting, place all the supplies and sterile milliQ Water needed for the PCR under the PCR hood and switch on the UV light for at least 20 min.

Tagged Primer Design and preparation

1 We use the eukaryotic 18SV4 primer set TAReukF1- TAReukR3 from Stoeck et al. 2010.

Primer	Target	Sense	Sequence 5'-3'	Length (pb)	Tm (°C)	Amplicon length (pb)	Source
TAReuk_F1 (V4f)	18S_Eukaryotes	Forward	cca gca scy gcg gta att cc	20	64	380	Stoeck et al.
TAReuk_R (V4r)	18S_Eukaryotes	Reverse	act ttc gtt ctt gat yra	18	48		2010

Citation: Sarah Romac Eukaryotes 18S-V4 rRNA Metabarcoding PCR protocol for NGS Illumina sequencing https://dx.doi.org/10.17504/protocols.io.bzucp6sw

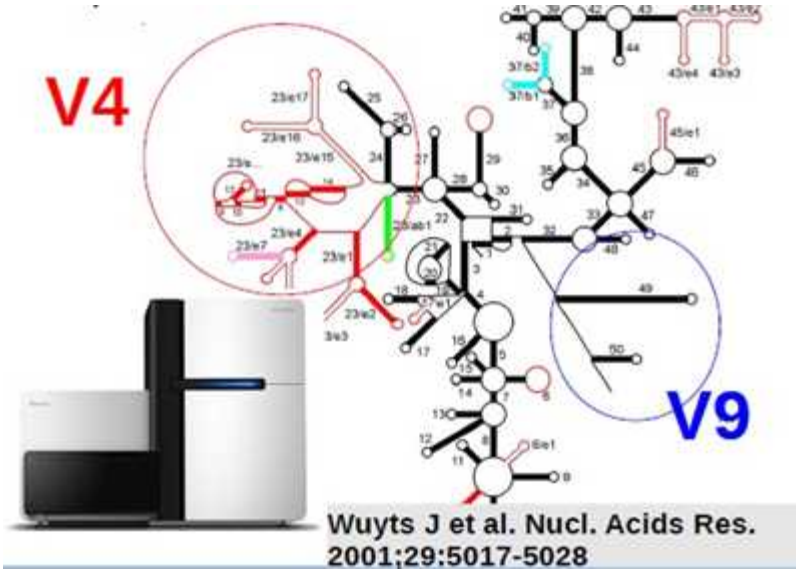


Figure 1 : Location of V4 part on the SSU structure. (Wuyts et al. 2001).

2

Step 2 includes a Step case.

Tagged-Primer Design for Fasteris platform

Tagged-Primer Design for GeT-PLaGE platform

step case

Tagged-Primer Design for Fasteris platform

Amplicons from each DNA sample are all pooled in a single microtube. Each pool of amplicons will be considered as a "library sample " or called "**Pooled Amplicons**" and loaded on a Miseq run.

To allow the latter separation of each sample in the Pooled Amplicon sample, each DNA is amplified using forward tagged primer built with a structure 5'-NNNN-MID-forwardprimer'. The reverse primer is not modified.

The tag, or Multiplex IDentifier (MID) is a unique short sequence of 7 or 8 bases compatible with the forward primer V4f and generated using the matrix oligoTag program (Coissac et al. 2012). We also added 4 N at the 5' extremity of the forward primer to help MID sequence conservation during the cluster synthesis step on the Flowcell.

Citation: Sarah Romac Eukaryotes 18S-V4 rRNA Metabarcoding PCR protocol for NGS Illumina sequencing https://dx.doi.org/10.17504/protocols.io.bzucp6sw

Primer	Sense	Tagged primer construction	Sequence 5'-3'	Amplicon length (pb)
V4f-Fasteris	Forward	5' –NNNN-MID-primer – 3'	NNNNMID###ccagcascygcggtaattcc	427
V4r-Fasteris	Reverse	5' – primer – 3'	actttcgttcttgatyra	

Figure 2 : Fasteris tagged-primer description.

Primer_Name	MIDnumber	MIDsequence 5'-3'
V4F_M8R0001	M8R0001	AACAACAA
V4F_M8R0004	M8R0004	ccaggtga
V4F_M8R0005	M8R0005	AGCATGCG
V4F_M8R0006	M8R0006	aatggagg
V4F_M8R0007	M8R0007	ttctcctg
V4F_M8R0008	M8R0008	CTTCTTCA
V4F_M8R0009	M8R0009	cgcaacag
V4F_M8R0010	M8R0010	AACAATGG
V4F_M8R0012	M8R0012	GAGTACTA
V4F_M8R0015	M8R0015	TATCACAT
V4F_M8R0016	M8R0016	gtcgctgt
V4F_M8R0017	M8R0017	ccgagatt
V4F_M8R0019	M8R0019	cgcaagca
V4F_M8R0020	M8R0020	CCAGTCAG
V4F_M8R0023	M8R0023	CTATAAGT
V4F_M8R0024	M8R0024	cttgacag
V4F_M8R0025	M8R0025	aacaccgt
V4F_M8R0026	M8R0026	ATGTATAA
V4F_M8R0028	M8R0028	TATCAGGA
V4F_M8R0029	M8R0029	gcgacaat
V4F_M8R0030	M8R0030	gtcggtca
V4F_M8R0031	M8R0031	AACGAGTG
V4F_M8R0032	M8R0032	aacacgta
V4F_M8R0034	M8R0034	GTGAATTA
V4F_M8R0035	M8R0035	TGATCCTA
V4F_M8R0036	M8R0036	CGCACCTT
V4F_M8R0038	M8R0038	ATACATGG
V4F_M8R0040	M8R0040	AACAGACA
V4F_M8R0041	M8R0041	tatccagg
V4F_M8R0042	M8R0042	ttgccacg
V4F_M8R0043	M8R0043	AGCCATGT
V4F_M8R0046	M8R0046	TTCCTCAT
V4F_M8R0047	M8R0047	agccgata
V4F_M8R0049	M8R0049	catggcca
V4F_M8R0050	M8R0050	aacaggag
V4F_M8R0051	M8R0051	tatcctca
V4F_M8R0052	M8R0052	cacttcaa
V4F_M8R0053	M8R0053	aattaagt
V4F_M8R0055	M8R0055	tgatgatt
V4F_M8R0056	M8R0056	tcgatcgg
V4F_M8R0059	M8R0059	ctatcgcg
V4F_M8R0061	M8R0061	tatcgata
V4F_M8R0065	M8R0065	aacatatg
V4F_M8R0066	M8R0066	atgtgccg
V4F_M8R0067	M8R0067	ttgcgcct
V4F_M8R0069	M8R0069	aattagta
V4F_M8R0070	M8R0070	acgtcacg
V4F_M8R0071	M8R0071	agcctctt
V4F_M8R0072	M8R0072	cggtcaca
V4F_M8R0073	M8R0073	tgatgtcg
V4F_M8R0074	M8R0074	ctatgaca
V4F_M8R0075	M8R0075	tatcggct
V4F_M8R0076	M8R0076	agccttaa
V4F_M8R0079	M8R0079	gtctatga
V4F_M8R0080	M8R0080	aacattat
V4F_M8R0083	M8R0083	atacgtca
V4F_M8R0084	M8R0084	aaccaacg
V4F_M8R0085	M8R0085	taatgcgt
V4F_M8R0086	M8R0086	tcgcagta
V4F_M8R0087	M8R0087	acagcata
V4F_M8R0090	M8R0090	tgattgat
V4F_M8R0091	M8R0091	aaccagat
V4F_M8R0093	M8R0093	gtctcgca
V4F_M8R0405	M8R0405	TCGCAAGG
V4F_M8R0409	M8R0409	AGGCTTCG
V4F_M8R0412	M8R0412	TCACGACG
V4F_M8R0413	M8R0413	ACATAGCG
V4F_M8R0416	M8R0416	GACCACTG
V4F_M8R0418	M8R0418	ACTGTTAG
V4F_M8R0430	M8R0430	GTGTGATA
V4F_M8R0431	M8R0431	CAGCGGTA
V4F_M8R0432	M8R0432	ACATCCAT
V4F_M8R0434	M8R0434	TTATCTGT
V4F_M8R0435	M8R0435	AAGCAATT
V4F_M8R0436	M8R0436	CAACCGCA
V4F_M8R0439	M8R0439	CGTAGTGG
V4F_M8R0636	M8R0636	gttctatt
V4F_M8R0640	M8R0640	ctggtatt
V4F_M8R0646	M8R0646	cgttcgtt
V4F_M8R0690	M8R0690	aataactt
V4F_M8R0694	M8R0694	atgacctt
V4F_M8R0702	M8R0702	tccagttg
V4F_M8R0703	M8R0703	aataccag
V4F_M8R0704	M8R0704	ctaagccg
V4F_M8R0706	M8R0706	catgagcg
V4F_M8R0710	M8R0710	gcatctag
V4F_M8R0801	M8R0801	tagttagg
V4F_M8R0809	M8R0809	cttatcgg
V4F_M8R0816	M8R0816	caagccgt
V4F_M8R0817	M8R0817	ttgaactg
V4F_M8R0818	M8R0818	acgcagct
V4F_M8R0820	M8R0820	tcctcgga
V4F_M8R0821	M8R0821	tgagatta
V4F_M8R0822	M8R0822	acgcatga
V4F_M8R0824	M8R0824	gtccacca
V4F_M8R0825	M8R0825	tgagcact
V4F_M8R0826	M8R0826	acaacaag
V4F_M8R0828	M8R0828	tcctgagg
V4F_M8R0830	M8R0830	ccacgttg
V4F_M8R0831	M8R0831	taagagtt
V4F_M8R0833	M8R0833	ctcggaat
V4F_M8R0834	M8R0834	agcataga
V4F_M8R0835	M8R0835	acgcgctt
V4F_M8R0836	M8R0836	cgatcatg
V4F_M8R0837	M8R0837	gtccgctt
V4F_M8R0838	M8R0838	aatcttgt
V4F_M8R0841	M8R0841	ccacttaa
V4F_M8R0843	M8R0843	gagtatct
V4F_M8R0844	M8R0844	aaggcgca
V4F_M8R0845	M8R0845	cttcctag
V4F_M8R0846	M8R0846	acgttgca
V4F_M8R0847	M8R0847	aactgtga
V4F_M8R0849	M8R0849	aatgctct
V4F_M8R0851	M8R0851	agcactag
V4F_M8R0853	M8R0853	ttgagcaa
V4F_M8R0854	M8R0854	taagctcg
V4F_M8R0856	M8R0856	tgcagtat
V4F_M8R0857	M8R0857	tgtgcgag
V4F_M8R0858	M8R0858	taggtgct
V4F_M8R0859	M8R0859	gtgcttgg
V4F_M8R0860	M8R0860	tgcgtgta
V4F_M8R0861	M8R0861	acaagtgg
V4F_M8R0862	M8R0862	tcgaacca
V4F_M8R0863	M8R0863	agcagcct
V4F_M8R0865	M8R0865	gacatcca
V4F_M8R0866	M8R0866	atggtacg
V4F_M8R0867	M8R0867	cagtatga
V4F_M8R0868	M8R0868	ttgagtcg
V4F_M8R0871	M8R0871	ctcacttg
V4F_M8R0873	M8R0873	tcgaatag
V4F_M8R0876	M8R0876	gccttcag
V4F_M8R0877	M8R0877	ataatcgt
V4F_M8R0879	M8R0879	tcgacaga
V4F_M8R0880	M8R0880	atggttat
V4F_M8R0882	M8R0882	acggattg
V4F_M8R0883	M8R0883	tgatacag
V4F_M8R0884	M8R0884	ttgatggt
V4F_M8R0885	M8R0885	gtcgcaag
V4F_M8R0886	M8R0886	tgtgtagg

Figure 3 : MID list adapted for 18S-V4 primer set.

3 Lyophilized Tagged-primers are obtained at Eurogentec, using the RP-Cartridge Gold purification.

Work always under the PCR hood.

Citation: Sarah Romac Eukaryotes 18S-V4 rRNA Metabarcoding PCR protocol for NGS Illumina sequencing https://dx.doi.org/10.17504/protocols.io.bzucp6sw

3.1 Elute dried primers at 100 µM with TE 1X sterile buffer under a PCR hood.

3.2 Primer dissolution is done for 15 min at room temperature under the hood. Short vortex and spin.

3.3 Primer working solutions are then prepared at 10 µM with sterile milliQ water molecular grade.

For each MID-primer, add 10 µL of 100 µM of Stock primer to 90 µL in a 1.5mL sterile microtube correctly labeled (MID###, concentration, date, operator).

3.4 Stock primers and primer working solutions are stored at -20°C.

PCR

4 PCR reactions are performed using the Taq polymerase **Phusion High-Fidelity Master Mix with GC buffer (Thermofisher, Cat No F-532 L)**.
This Taq has a good proof-reading and its buffer allows amplification of high GC templates.

Keep the same annealing temperature as the one is used with the usual 18S-V4 primer set.

First test each of your taggeg-primer sets on a positive and a negative control following steps 5, 6, 7 and 8.

Then you can perform DNA sample amplification following next steps.

5 **PCR plate's plan :**

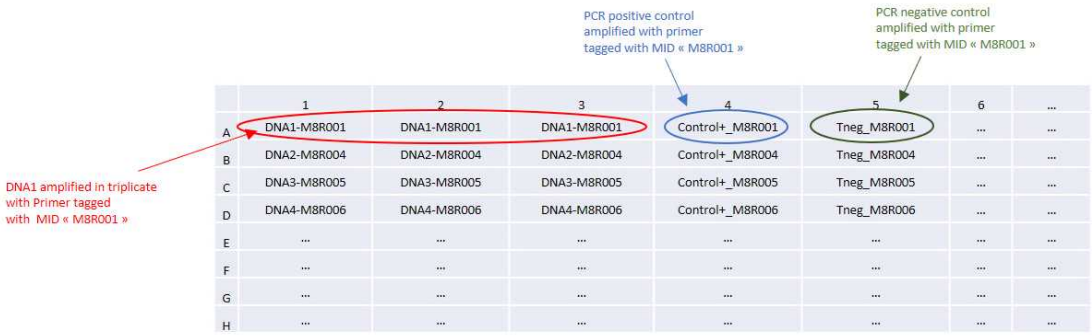
Each DNA sample (DNA1, DNA2, DNA3...) will be amplified with its own tagged-MID-primer (V4f-M8R001, V4f-M8R004, V4f-M8R005...). **So there will be as many PCR mix preparations as there are DNA samples to amplify.**

In order to get enough material (50 ng), triplicate the PCR reactions on each DNA sample (you will pool them after).

One positive control and one negative control will be added for each PCR mix preparation.

PCR reactions are prepared on a semi-skirted 96-wells PCR plate (like AB-0900 PCR plate).

Citation: Sarah Romac Eukaryotes 18S-V4 rRNA Metabarcoding PCR protocol for NGS Illumina sequencing
https://dx.doi.org/10.17504/protocols.io.bzucp6sw



6 PCR Mix preparation :

6.1 Prepare the Master Mix :

Designation	Final concentration	Volume (µL)
GC Mastermix Phusion 2x	1x	12,5
Primer forward 10µM	0,35 µM	1
Primer reverse 10µM	0,35 µM	1
DMSO 100%	3%	0,75
H2O Ultra Pure		8,75
DNA template 5ng/µL	5ng	1
Total volume		25

6.2 Dispence 24 µL of each tagged-MID-primer - PCR mix preparation per well under the PCR hood, and then add 1 (5ng) µL of template on the bench.

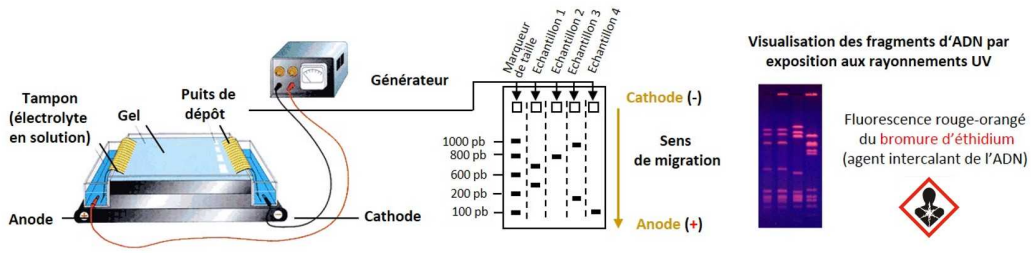
7 PCR Programm :

Citation: Sarah Romac Eukaryotes 18S-V4 rRNA Metabarcoding PCR protocol for NGS Illumina sequencing https://dx.doi.org/10.17504/protocols.io.bzucp6sw

Initial Denaturation	30sec @ 98°C	
Denaturation	10sec @ 98°C	x 12cycles
Annealing	30sec @ 53°C	
Elongation	30sec @ 72°C	
Denaturation	10sec @ 98°C	x 18cycles
Annealing	30sec @ 48°C	
Elongation	30sec @ 72°C	
Final Elongation	10min @ 72°C	
Storage	∞ @ 4°C	

In order to reduce the artificial building of chimeras during the PCR process, you should reduce the # of cycles to a minimum : 20, 25, max 30 (chimerization occurs principally during the plateau-phase of the PCR reaction).

8 Check the quality of all the PCR products on an 1.2 % agarose gel :



Be very cautious with Ethidium Bromide manipulation!

FDS Ethidium Bromide.pdf

8.1 Prepare 1,2 % agarose gel in TAE 0.5x buffer :

- In a Becher, put 1.2g agarose (Interchim, ref 31272L) in 100mL TAE 0.5x buffer (TAE prediluted in milliQ water from TAE 10x-Thermofisher Scientific, ref 15558042).

- Heat under total dissolution of the agarose powder (you can use a microwave or a stirer plate).

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- Add one drop of Ethidium Bromide (Eurobio, ref GEPBET02AF).

- Prepare casting tray with combs according to your number of PCR products to check. (Biorad, ref 1704484)

Biorad_Gel Caster.pdf

- Poor gel in the casting tray and check there are no bubbles.

- Let solidify for 20 min.

8.2 Sample loading and electrophoresis conditions :

Prepare the loading samples :
In a semi-skirted 96-wells PCR plate (like AB-0900 PCR plate), mix 5µL of each sample with 1µL of loading buffer 6x (Thermofisher Scientific, ref R0611).

Place solidified gel in the proper orientation (electrophoresis occurs from cathode to anode).

Load :
- PCR products : 6µL ;
- Smartladder 200 to 10 000 bp : 3µL ; (Eurogentec, ref MW-1700-10).

Close the caster and connect it to the generator (Bio-Rad, ref 1645050).

Let run the electrophoresis at 110 V for 45 min.

8.3 Amplification result observation :

After migration, observe amplification results under UV light using an Imager (for instance : ImageQuant LAZ4000, GE Healthcare).

LAS 4000 User manual.pdf

Amplifications worked very well if :
- Negative control has not amplified;
- Positive control has amplified;
- Amplifications have band at the good size (427 pb), no smear.

Store the amplicons at -20°C until PCR purification.

PCR product purification

Citation: Sarah Romac Eukaryotes 18S-V4 rRNA Metabarcoding PCR protocol for NGS Illumina sequencing https://dx.doi.org/10.17504/protocols.io.bzucp6sw

9 PCR products are purified using the purification kit: **NucleoSpin® PCR Clean-Up (Macherey-Nagel, cat. nb 740609.50 or 740609.250)**.

Store the kit at room temperature.

Instruction-NucleoSpin-Gel-and-PCR-Clean-up.pdf

10 Prepare Purification Run Table and pool the triplicate PCR into a single microtube with appropriate labelling (sample, target and tag-MID nb, PCR date).

Sample Code	Primer MID	PCR plate and wells	V pool amplicons (µL)	V NT buffer µL	V column µL	V NE elution µL
DNA1-M8R001	V4F_M8R0001	PCR_###_wells A1 to A3	60	120	180	22
DNA2-M8R004	V4F_M8R0004	PCR_###_wells B1 to B3	60	120	180	22
DNA3-M8R005	V4F_M8R0005	PCR_###_wells C1 to C3	60	120	180	22
DNA4-M8R006	V4F_M8R0006	PCR_###_wells D1 to D3	60	120	180	22
...	...	...	...	...	...	...

11 Mix 1 vol of sample with 2 volumes of buffer NT.
Follow the instructions of manufacturer (mentioned in the Step 9), except for the elution step.

Elution Step :

- Place the NucleoSpin PCR Clean-Up Column into a clean 1.5mL microtube correctly labeled (sample, target tagged-MID, date).
- Add 22 µL buffer NE preheated at 65°C directly onto the column and incubate 5 min @ 65°C.
- Centrifuge 1 min @ 11 000 g.

Store the purified PCR products at -20°C or directly do the quantification.

PCR products quantification

12 PCR products are quantified using the quantification kit: Quant-iT™ PicoGreen® dsDNA reagent *2000 assays* (Invitrogen, cat nb P7581) and a Fluorometer Plate readerFluorometer Plate reader, following the manufacturer's protocol.

Quant-it Picogreen dsDNA kit.pdf

Final Pool Amplicons preparation

13 After quantification of the PCR products, amplicons will be pooled before shipment to the

Citation: Sarah Romac Eukaryotes 18S-V4 rRNA Metabarcoding PCR protocol for NGS Illumina sequencing https://dx.doi.org/10.17504/protocols.io.bzucp6sw

NGS Fasteris sequencing platform.

One final tube (called **"Pooled Amplicons"**) will contain all pooled amplicons at equimolar concentration, ready for the library preparation. The amounts and volume required by Fasteris are : **1µg of equimolar amplicon pool in 30 µl (so Pool Amplicons concentration should be >35 ng/µL) .**

The volume of each amplicon that will added in the tube "Pooled Amplicons" is calculated based on their average concentration as follows :

Calculation table for Pool Amplicons preparation :

DNA1 has a different concentration than DNA2, DNA3...

But DNA1 needs to have the same mass than DNA2, DNA3... in the « Pool Amplicon »

Volume calculated of DNA1 to mix in the same microtube with DNA2, DNA3... in the « Pool Amplicon »
DNA1, DNA2, DNA3... wil be each added with a different volume

Sample name	Primer_MID	nb lect att.	Average Concentration ng/µL	Amount in the final tube ng	Volume in the final tube µL
DNA1-M8R001	V4F_M8R0001	150000	c1	m	=(m/c1)
DNA2-M8R004	V4F_M8R0004	150000	c2	m	=(m/c2)
DNA3-M8R005	V4F_M8R0005	150000	c3	m	=(m/c3)
DNA4-M8R006	V4F_M8R0006	150000	c4	m	=(m/c4)
...	...	...	...	m	=(m/c...)
...	...	...	...	m	=(m/c...)
...	...	...	...	m	=(m/c...)
...	...	...	...	m	=(m/c...)
Total number of reads for Miseq		15-25 000 000		= Total amount ng (>1µg)	= Total volume µL (<30 µL)
			Pool Amplicons ng/µL		35 ng/µL

Volume, amount and so concentration required by Fasteris

If the final volume of Pooled Amplicons is higher than 30 µL, (so concentration inferior to 35ng/µL), an additionnal concentration step will be necessary. For this we used the purification kit: **NucleoSpin® PCR Clean-Up (Macherey-Nagel, cat. nb 740609.50 or 740609.250)**.

Step 13 includes a Step case.

Pool Amplicons Concentration

Pool Amplicons Quantification and Quality Checking

step case

Pool Amplicons Concentration

This step is performed only if your Pool Amplicons has the following parameters >30µL and <35 ng/µL.
We use the kit **NucleoSpin® PCR Clean-Up (Macherey-Nagel, cat. No 740609.50)**.

We decided to fix the **Final Concentration at 50 ng/µL**, to be sure to be in excess.

Citation: Sarah Romac Eukaryotes 18S-V4 rRNA Metabarcoding PCR protocol for NGS Illumina sequencing https://dx.doi.org/10.17504/protocols.io.bzucp6sw

1. Prepare your Concentration File :

Sample name	Initial Conc. Ci ng/µL	Vi µL	V NT buffer µL	Vcolumn µL	Velution µL	Final Conc. Cf ng/µL
PoolAmplicon 1	Ci	Vi	= 2 *Vi	= Vi + VNT	= (Ci*Vi)/Cf	50

The elution volume is calculated depending of the Initial Concentration and Initial Volume of the Pool Amplicons sample.

2. Add 2 *Vi µL of NT buffer and follow the recommendations of the manufacturer as mentionned in
Step 9
, except for the Elution Step.

Elution Step :

- Place the NucleoSpin PCR Clean-Up Column into a clean 1.5mL microtube correctly labeled (Pool Amplicon name, Quotation nb given by Fasteris, date).
- Add
Vf of buffer NE (calculated in your table above)
preheated at 65°C directly onto the column and incubate 1 min @ room temperature.
- Centrifuge 1 min @ 11 000 g.

Store the concentrated Pool Amplicons at -20°C.

14 After the preparation of the Pooled Amplicons (and concentration if needed), the final concentration is checked by quantification using **Qubit 4 Fluorometer** (Invitrogen) with the kit **Qubit 1x dsDNA HS Assay** (Invitrogen, Thermofisher Scientific cat. No Q33230).

MAN0019617_Qubit_1X_dsDNA_BR_Assay_UG.pdf

15 If possible, check the final Pooled Amplicons quality on a Bioanalyzer using the kit **Agilent DNA 1000** (Agilent Technologies, Cat. No 5067-1504).

G2938-90014_DNA1000Assay_KG.pdf

16 Store the Pooled Amplicons at -80°C until the shipment to Fasteris.

Citation: Sarah Romac Eukaryotes 18S-V4 rRNA Metabarcoding PCR protocol for NGS Illumina sequencing https://dx.doi.org/10.17504/protocols.io.bzucp6sw

Shipment Conditions to Fasteris

17 Pooled Amplicons must be shipped via Dry Ice to the following address :
FASTERIS SA
NGS Services
Chemin du Pont-du-Centenaire 109
CH-1228 Plan-les-Ouates
Switzerland

Additonnal documents to include in the package (that must also be sent by e-mail to Fasteris NGS services (ngs@fasteris.com) :
- Quotation number Q##### ;
- Order form ;
- Purchase oder edited by your company.

Don't forget to add a Pro Forma Invoice in the package.

Citation: Sarah Romac Eukaryotes 18S-V4 rRNA Metabarcoding PCR protocol for NGS Illumina sequencing https://dx.doi.org/10.17504/protocols.io.bzucp6sw