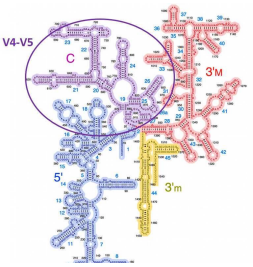
Sep 20, 2024 Version 2

Prokaryotes/Eukaryotes 16S-V4V5 rRNA Metabarcoding PCR protocol for NGS Illumina sequencing V.2



DOI
dx.doi.org/10.17504/protocols.io.36wgq42o3vk5/v2

Sarah Romac[1]

[1]UMR7144-Roscoff Marine Station

Ecology of Marine Plankto… Protist Research to Optimi… 1 more workspace

Sarah Romac
UMR7144-Roscoff Marine Station

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.36wgq42o3vk5/v2

Protocol Citation: Sarah Romac 2024. Prokaryotes/Eukaryotes 16S-V4V5 rRNA Metabarcoding PCR protocol for NGS Illumina sequencing. **protocols.io https://dx.doi.org/10.17504/protocols.io.36wgq42o3vk5/v2**Version created by **Sarah Romac**

Protocol status: Working
We use this protocol and it's working

Created: November 09, 2021

Last Modified: September 20, 2024

Protocol Integer ID: 108094

Keywords: PCR, NGS, metabarcoding, prokaryotes, 16S-V4V5

Abstract

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

Here we describe the 16S-V4V5 DNA amplification method applied for both prokaryote and eukaryotes metabarcoding analyses using Illumina Miseq technology. This protocol has been used in many projects studying prokaryotic and eukaryotic diversity (TARA-OCEANS 2009-2013, TARA-PACIFIC 2016-2018), and prokaryote monitoring projects (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).

Guidelines

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.

Materials

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.

Before start

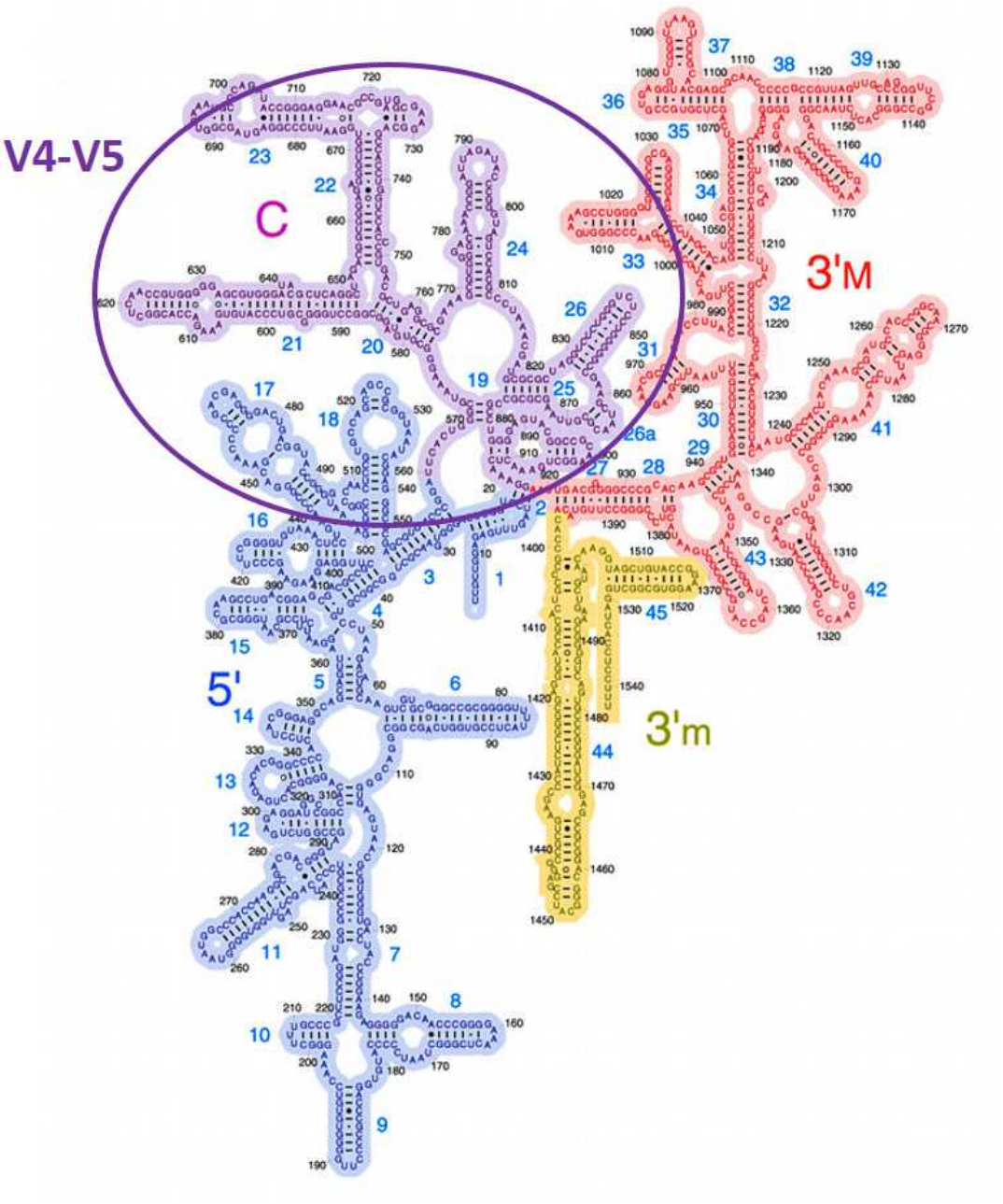
Do aliquots of 1 mL of sterile milliQ water.

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 prokaryotic 16SV4V5 primer set 515fY- 926r from Parada et al. 2016.

Primer	Target	Sense	Sequence 5'-3'	Length bp	Tm °C	Amplicon length bp	Source
515f-Y	16S_Prokaryotes	Forward	GTGYCAGCMGCCGCGGTAA	19	63	411	Parada et al. 2016
926r	16S_Prokaryotes	Reverse	CCGYCAATTYMTTTRAGTTT	20	63		



Location of the V4V5 part on the 16S structure.

2

STEP CASE

Tagged-Primer Design for Fasteris platform 24 steps

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 2x250 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.

Primer	Sense	Tagged primer construction	Sequence 5'-3'	Amplicon Length (pb)
515f-Y-Fasteris	Forward	5' –NNNN-MID-primer – 3'	NNNNMID###GTGYCAGCMGCCGCGGTAA	423
926r-Fasteris	Reverse	5' – primer – 3'	CCGYCAATTYMTTTRAGTTT	

Figure 2 : Fasteris tagged-primer description.

Primer Name	MIDnumber	MIDsequence
515fY_M8R0001	M8R0001	AACAACAA
515fY_M8R0004	M8R0004	CCAGGTGA
515fY_M8R0005	M8R0005	AGCATGCG
515fY_M8R0006	M8R0006	AATGGAGG
515fY_M8R0007	M8R0007	TTCTCCTG
515fY_M8R0008	M8R0008	CTTCTTCA
515fY_M8R0009	M8R0009	CGCAACAG
515fY_M8R0010	M8R0010	AACAATGG
515fY_M8R0011	M8R0011	GGCGTTAT
515fY_M8R0012	M8R0012	GAGTACTA
515fY_M8R0015	M8R0015	TATCACAT
515fY_M8R0016	M8R0016	GTCGCTGT
515fY_M8R0017	M8R0017	CCGAGATT
515fY_M8R0018	M8R0018	GAGACGAG
515fY_M8R0019	M8R0019	CGCAAGCA
515fY_M8R0020	M8R0020	CCAGTCAG
515fY_M8R0024	M8R0024	CTTGACAG
515fY_M8R0025	M8R0025	AACACCGT
515fY_M8R0026	M8R0026	ATGTATAA
515fY_M8R0028	M8R0028	TATCAGGA
515fY_M8R0029	M8R0029	GCGACAAT
515fY_M8R0030	M8R0030	GTCGGTCA
515fY_M8R0031	M8R0031	AACGAGTG
515fY_M8R0032	M8R0032	AACACGTA
515fY_M8R0034	M8R0034	GTGAATTA
515fY_M8R0035	M8R0035	TGATCCTA
515fY_M8R0038	M8R0038	ATACATGG
515fY_M8R0041	M8R0041	TATCCAGG
515fY_M8R0042	M8R0042	TTGCCACG
515fY_M8R0043	M8R0043	AGCCATGT
515fY_M8R0046	M8R0046	TTCCTCAT
515fY_M8R0047	M8R0047	AGCCGATA
515fY_M8R0049	M8R0049	CATGGCCA
515fY_M8R0050	M8R0050	AACAGGAG
515fY_M8R0051	M8R0051	TATCCTCA
515fY_M8R0052	M8R0052	CACTTCAA
515fY_M8R0053	M8R0053	AATTAAGT
515fY_M8R0055	M8R0055	TGATGATT
515fY_M8R0056	M8R0056	TCGATCGG
515fY_M8R0057	M8R0057	GGCTGATG
515fY_M8R0059	M8R0059	CTATCGCG
515fY_M8R0061	M8R0061	TATCGATA
515fY_M8R0064	M8R0064	GGCTGTCT
515fY_M8R0065	M8R0065	AACATATG
515fY_M8R0066	M8R0066	ATGTGCCG
515fY_M8R0067	M8R0067	TTGCGCCT
515fY_M8R0069	M8R0069	AATTAGTA
515fY_M8R0070	M8R0070	ACGTCACG
515fY_M8R0071	M8R0071	AGCCTCTT
515fY_M8R0072	M8R0072	CGGTCACA
515fY_M8R0073	M8R0073	TGATGTCG
515fY_M8R0074	M8R0074	CTATGACA
515fY_M8R0075	M8R0075	TATCGGCT
515fY_M8R0076	M8R0076	AGCCTTAA
515fY_M8R0079	M8R0079	GTCTATGA
515fY_M8R0080	M8R0080	AACATTAT
515fY_M8R0082	M8R0082	GGCTTGAA
515fY_M8R0083	M8R0083	ATACGTCA
515fY_M8R0084	M8R0084	AACCAACG
515fY_M8R0085	M8R0085	TAATGCGT
515fY_M8R0086	M8R0086	TCGCAGTA
515fY_M8R0087	M8R0087	ACAGCATA
515fY_M8R0088	M8R0088	GGTAACCT
515fY_M8R0090	M8R0090	TGATTGAT
515fY_M8R0091	M8R0091	AACCAGAT
515fY_M8R0093	M8R0093	GTCTCGCA
515fY_M8R0094	M8R0094	ATGTTCTA
515fY_M8R0095	M8R0095	GCGCTATG
515fY_M8R0096	M8R0096	GCGCTGAA
515fY_M8R0097	M8R0097	AATGGTTA
515fY_M8R0098	M8R0098	TTGCTTAA
515fY_M8R0099	M8R0099	AACCATTA
515fY_M8R0100	M8R0100	CTTGTGTA
515fY_M8R0101	M8R0101	TCGCCTGT
515fY_M8R0102	M8R0102	CTATTCAT
515fY_M8R0103	M8R0103	TGTTGTTA
515fY_M8R0105	M8R0105	AACCGAGT
515fY_M8R0106	M8R0106	AGACTAAT
515fY_M8R0107	M8R0107	CAGAGAAG
515fY_M8R0108	M8R0108	TGCAATCG
515fY_M8R0109	M8R0109	ACGTGTTA
515fY_M8R0110	M8R0110	TATGAATG
515fY_M8R0111	M8R0111	ATAGAACT
515fY_M8R0112	M8R0112	TTAACCAG
515fY_M8R0113	M8R0113	TAATTGTA
515fY_M8R0114	M8R0114	CGCGATGA
515fY_M8R0115	M8R0115	ATTAATAT
515fY_M8R0116	M8R0116	CAGAGCCT
515fY_M8R0118	M8R0118	GCGGCTTA
515fY_M8R0119	M8R0119	TATGTACA
515fY_M8R0123	M8R0123	TATGATAA
515fY_M8R0126	M8R0126	GAACATGA
515fY_M8R0127	M8R0127	AACCTCAG
515fY_M8R0128	M8R0128	TATGCAAT
515fY_M8R0129	M8R0129	TGCACTGA
515fY_M8R0130	M8R0130	ACAACCGA
515fY_M8R0131	M8R0131	TTGGCAGT
515fY_M8R0133	M8R0133	CTATACTG
515fY_M8R0134	M8R0134	CGCTAAGG
515fY_M8R0135	M8R0135	ACTAAGAG

Figure 3 : MID list adapted for 16S-V4V5 primer set.

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

Work always under the PCR hood.

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 16S-V4V5 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.

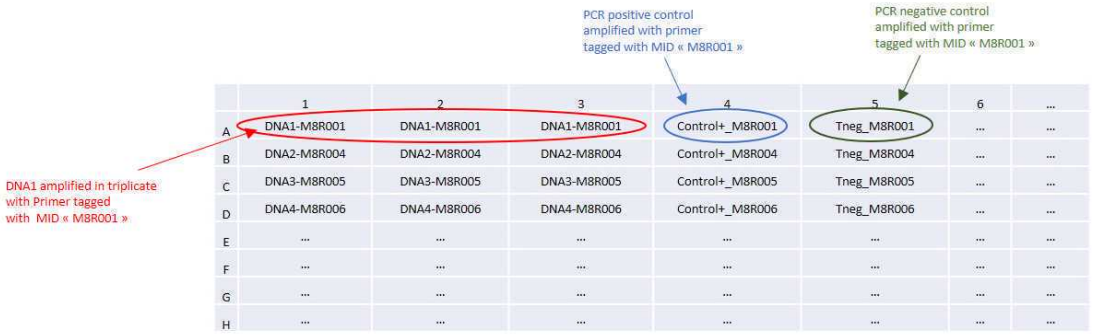
5 **PCR plate's plan :**

Each DNA sample (DNA1, DNA2, DNA3...) will be amplified with its own tagged-MID-primer (515fY-M8R001, 515fY-M8R004, 515fY-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).



	1	2	3	4	5	6	...
A	DNA1-M8R001	DNA1-M8R001	DNA1-M8R001	Control+_M8R001	Tneg_M8R001	...	...
B	DNA2-M8R004	DNA2-M8R004	DNA2-M8R004	Control+_M8R004	Tneg_M8R004	...	...
C	DNA3-M8R005	DNA3-M8R005	DNA3-M8R005	Control+_M8R005	Tneg_M8R005	...	...
D	DNA4-M8R006	DNA4-M8R006	DNA4-M8R006	Control+_M8R006	Tneg_M8R006	...	...
E	...	...	...	...	...	...	...
F	...	...	...	...	...	...	...
G	...	...	...	...	...	...	...
H	...	...	...	...	...	...	...

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 If you work on environmental sample :

Dispense 24 µL of each tagged-MID-primer PCR mix preparations per well under the PCR hood, and then add 1 µL of the DNA template on the bench.

If you work on strain sample :
Dispense 21 µL of each tagged-MID-primer PCR mix preparations per well under the PCR hood (so reduce the volume of H2O milliQ in the Master Mix), and then add 4 µL of the DNA template on the bench to get 5-10ng in the MasterMix.

7 **PCR Programm :**

Initial Denaturation	30sec @ 98°C	
Denaturation	10sec @ 98°C	x 25cycles
Annealing	30sec @ 53°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, the # of cycles must be reduced to a minimum : 25, max 30 (chimeras formation mainly during the plateau-phase of the PCR reaction).

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

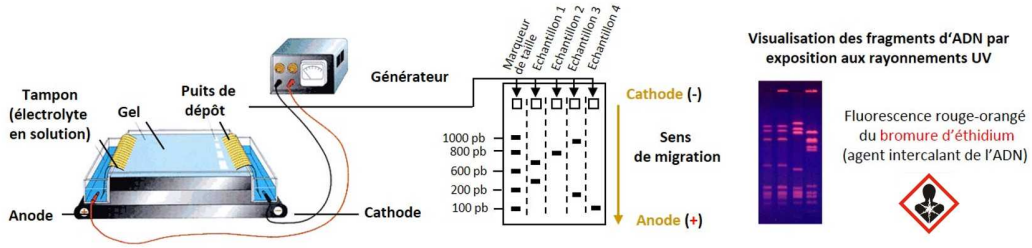


Figure 4 : Principle of DNA electropheris step (in French).

Safety information

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).

- 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).

FILE 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 (423 pb), no smear.
- It is possible to observe a second band 200 bp longer than the target. This is due to the presence of eukaryotes in the sample.

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

PCR product purification

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.

FILE Instruction-NucleoSpin-Gel-and-PCR…

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	515fY_M8R0001	PCR_###_wells A1 to A3	60	120	180	22
DNA2-M8R004	515fY_M8R0004	PCR_###_wells B1 to B3	60	120	180	22
DNA3-M8R005	515fY_M8R0005	PCR_###_wells C1 to C3	60	120	180	22
DNA4-M8R006	515fY_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.

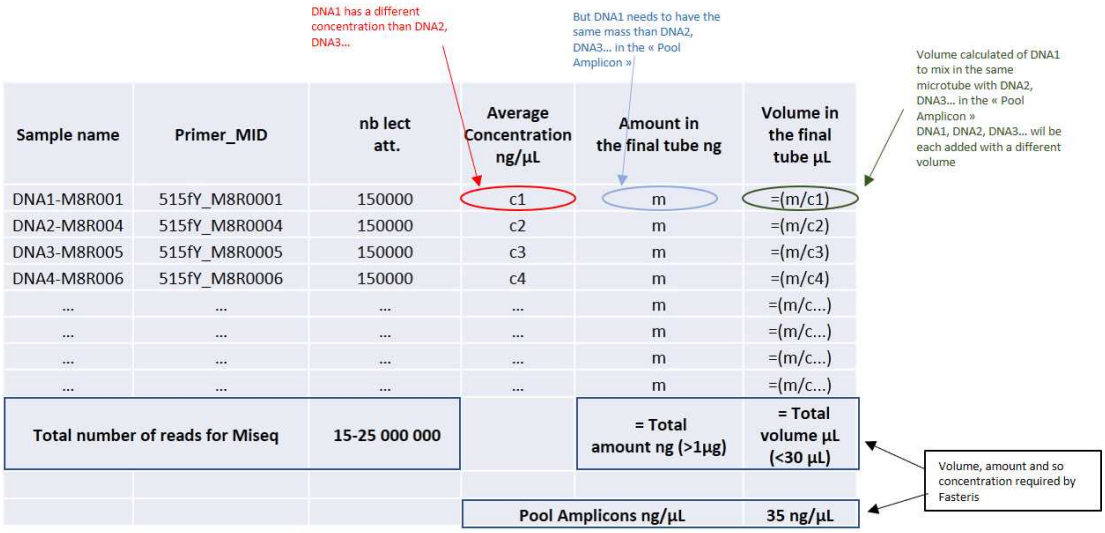
Quant-it Picogreen dsDNA kit.pdf

13 After quantification of the PCR products, amplicons will be pooled before shipment to the 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 Pooled Amplicons preparation :



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	515fY_M8R0001	150000	c1	m	=(m/c1)
DNA2-M8R004	515fY_M8R0004	150000	c2	m	=(m/c2)
DNA3-M8R005	515fY_M8R0005	150000	c3	m	=(m/c3)
DNA4-M8R006	515fY_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

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 CASE

Pooled Amplicons Concentration 4 steps

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

The **Final Concentration is fixed at 50 ng/µL**, to be sure to be in excess.

Prepare the 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 on the Initial Concentration and Initial Volume of the Pooled Amplicons.

2. Add 2 *Vi µL of NT buffer and follow the recommendations of the manufacturer as mentioned 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 Pooled Amplicons at -20°C.

Pooled Amplicons Quantification and Quality Checking

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).

FILE MAN0019617_Qubit_1X_dsDNA_BR…

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

FILE G2938-90014_DNA1000Assay_KG.pdf

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

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 to your package.