LifeWatch – MoBiLab Report

Caterina Manzari, Claudia Lionetti, Anna Maria D'Erchia, Graziano Pesole, Institute of Biomembranes and Bioenergetics, Consiglio Nazionale delle Ricerche, Bari, Italy

Contact: *g.pesole@ibbe.cnr.it*

In Table 1 samples received from OSD in October 2014.

OSD ID number	Osd event	site name	Site Coordinator	sampling depth (m) - '0,1' refers to surface	protocol	DNA concentration (ng/µl)	volume of DNA sample (µl)	which type of filter?
2	**June 2014**	Roscoff SOMLIT	Christian Jeanthon	0,1	B	**0,9**	100	other
4	**June 2014**	Naples	Adriana Zingone	0,1	B	**4,2**	100	other
6	**June 2014**	Blanes	Josep M Gasol	0,1	B	**5,6**	200	other
9	**June 2014**	SPOTS	Jed Fuhrman	0,1	B	**9,7**	200	other
14	**June 2014**	Banyuls SOMLIT	Ian Salter	2	B	**2,9**	200	other
22	**June 2014**	Solemio-SOMLIT (Marseille)	Dominique Lefevre and Patrick Raimbault	0,1	B	**12,5**	200	other
30	**June 2014**	Finland/Tvärmin ne (DEVOTES)	Anke	0,1	B	**11,2**	200	other
37	**June 2014**	Florida Coral 1 - Port Everglades	Chris and Nathalie	1	B	**11,9**	200	other

39	**June 2014**	South Carolina 1 - Charleston Harbour	Chris and Nathalie	0,1	B	**26,3**	200	other
43	**June 2014**	California Scripps	Kelly & Nathalie	10	B	**8**	200	other
49	**June 2014**	Slovenia (Vida)	Valentina Turk	0,1	B	**12,1**	200	other
60	**June 2014**	South Carolina 2 - North Inlet	Marie Delorenzo and Nathalie	0,1	B	**50,5**	200	other
71	**June 2014**	Otago	Federico Baltar	0,1	B	**12,8**	200	other
72	**June 2014**	Boknis Eck, Baltic Sea	Carolin Löscher and Hermann Bange	0,1	B	**21,8**	200	other
99	**June 2014**	C1 North Adriatic Italy	Paola Del Negro, Bruno Cataletto, Mauro Celussi, Francesca Malfatti	0,1	B	**16,2**	200	other
123	**June 2014**	Shikmona	Eyal Rahav	4	B	**20,3**	200	other
124	**June 2014**	Osaka Bay	Takashi Yoshida, Hiroyuki Ogata	5	B	**4,2**	200	other
132	**June 2014**	Sdot YAM	Noga Stambler	0,1	B	**6,5**	200	other
141	**June 2014**	Raunefjorden	Lise Øvreås	5	B	**7,1**	200	other
142	**June 2014**	Gray's Reef National Marine Sanctuary	Sarah Fangman & Marc Frischer	0,1	B	**2,3**	200	other
143	**June 2014**	Skidaway Institute of Oceanography	Marc Frischer	0,1	B	**32**	200	other
149	**June 2014**	Laguna Rocha Norte	Dr. Cecilia Alonso	0,1	B	**13,9**	200	other
150	**June 2014**	Laguna Rocha Sur	Dr. Cecilia Alonso	0,1	B	**16,2**	200	other

151	**June 2014**	South Atlantic Microbial Observatory	Dr. Cecilia Alonso	0,1	B	**24**	200	other
152	**June 2014**	Compass Buoy Station - Bedford Basin	Jennifer Tolman	1	B	**18,7**	200	other
3	**June 2014**	Helgoland	Anna / Antje Wichels	0,1	B	**16,8**	200	sterivex
54	**June 2014**	Maine Booth Bay	Nicole Poulton	1	B	**48,5**	200	sterivex
55	**June 2014**	Maine Damariscotta River	Nicole Poulton	0,1	B	**27,3**	200	sterivex
76	**June 2014**	Italy - Foglia	Antonella Penna	0,1	B	**68,1**	200	sterivex
77	**June 2014**	Italy - Metauro	Antonella Penna	0,1	B	**65,6**	200	sterivex
80	**June 2014**	Greenland - Young Sound	Eric Collins	2	B	**3,8**	200	sterivex
146	**June 2014**	Fram Strait	Katja Metfies, Christina Bienhold	0,1	B	**20,3**	200	sterivex
159	**June 2014**	Brest-SOMLIT	Stéphane L'Haridon	0,1	B	**32,3**	200	sterivex

Table 1. Samples received from OSD in October

In addition to the samples listed in Table 1, the shipping box also contained two additional unclassified tubes, with no sampling information:

- OSD **142*** Grays Ref Euk
- OSD **4** Sterivex Naples

Both samples were processed with the other.

SUMMARY

First of all, the 35 metagenomic samples were spectrophotometrically quantified to confirm the actual concentration of DNA using Nanodrop 2100.

For the library preparation, the Illumina Nextera's protocol (Nextera DNA sample preparation guide, Illumina) was modified to obtain the 18S V4 and V9 amplicon libraries for sequencing on the Illumina MiSeq platform (Kozich et al. 2013; Manzari et al. 2014).

The protocol was based on two amplification steps: the first amplification was carried out with V4 or V9 universal primers with a 5' end specific overhang (**Nextera® transposase sequences**, Illumina customer sequence letter August 2014); the second amplification was carried out using a mixture of the Nextera index primers and Illumina P5 and P7 primers.

Among the 35 samples:

- Samples 4, 142, 142* were not amplificable for V9_18S_rRNA region
- Samples 4, 142, 142*, 6, 151 were not amplificable for V4_18S_rRNA region.

The electrophoretic profile of these five non-amplificable metagenomic samples (4, 142, 142*, 6, 151) showed high level of DNA degradation.

LIBRARY PREPARATION

In the first PCR, V4 and V9 regions of 18S rRNA gene were amplified using specific primer pairs for the regions of interest with Nextera transposase overhang sequences attached at the 5'end (Table 2).

Amplification was performed using the Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Inc., New England Biolabs) in a Mastercycler Thermal Cycler (Eppendorf, Hamburg, Germany). Each reaction mixture contained 2.5 ng or 5 ng of extracted DNA (for V9 and V4 region, respectively), 1X Buffer HF, 0.2 mM dNTPs, 0.5 μM of each primer, and 1U Phusion DNA Polymerase in a final volume of 25 μl. The cycling parameters for PCR were standardized as follows: initial denaturation 98 °C for 30 s, followed by 10 cycles of denaturation at 98 °C for 10 s, annealing at 44 or 56 °C (V4 and V9 region, respectively) for 30 s, extension at 72 °C for 15 s, and subsequently 15 cycles of denaturation at 98 °C for 10 s, annealing at 62 °C for 30 s, extension at 72 °C for 15 s, with a final extension step of 7 min at 72 °C (Table 3 and 4).

All PCRs were performed in the presence of a negative control (Molecular Biology Grade Water, RNase/DNase-free water). The PCR products (around 270 pb for V9 and 470 bp for V4) were visualized on 1.2 % agarose gel and purified using the AMPure XP Beads at a concentration of 1.2X vol/vol (Agencourt Bioscience Corporation, Beverly, Massachusetts).

Primers used for Amplification of V4_18S_rRNA	
V4_18SNext.For	5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG[**CCAGCASCYGCGGTAATTCC**]*3'
V4_18SNext.Rev	5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG[**ACTTTCGTTCTTGATYRATGA**]*-3'
Primers used for Amplification of V9_18S_rRNA	
V9_18SNext.For	5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG[**TTGTACACACCGCCCGTCGC**]*3'
V9_18SNext.Rev	5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG[**CCTTCYGCAGGTTCACCTAC**]*-3'

Table 2. Primer used for amplification of V4_18S_rRNA and V9_18S_rRNA regions.
**universal V4 and v9 priming sequences in bold*

Amplification of V4_18S_rRNA Region

PCR Reaction	**DNA template**	2.5 ng
	5X HF Buffer	1X
	dNTPs	f.c. 200 µM
	Primer Forward	f.c. 0.5 µM
	Primer Reverse	f.c. 0.5 µM
	H2O nuclease free	-
	Final Volume	25 µl

	Step	***temperature***	***Time***
Thermal Cycles	**Initial Denaturation**	98°C	30 seconds
	10 cycles	98°C	10 seconds
		44°C	30 seconds
		72°C	15 seconds
	15 cycles	98°C	10 seconds
		62°C	30 seconds
		72°C	15 seconds
	Final extention	72°C	7 minutes
	Hold	4-10°C	

Table 3. Amplification of V4_18S_rRNA Region: 1st PCR conditions

Amplification of V9_18S_rRNA Region		
PCR Reaction	**DNA template**	5 ng
	5X HF Buffer	1X
	dNTPs	f.c. 200 µM
	Primer Forward	f.c. 0.5 µM
	Primer Reverse	f.c. 0.5 µM
	H2O nuclease free	-
	Final Volume	25 µl

	Step	***temperature***	***Time***
Thermal Cycles	**Initial Denaturation**	98°C	30 seconds
	10 cycles	98°C	10 seconds
		56 °C	30 seconds
		72°C	15 seconds
	15 cycles	98°C	10 seconds
		62°C	30 seconds
		72°C	15 seconds
	Final extention	72°C	7 minutes
	Hold	4-10°C	

Table 4. Amplification of V9_18S_rRNA Region: 1st PCR conditions

The purified amplicons were used as templates in the second PCR round, which was performed with the Nextera indices priming sequences as required by the dual index approach reported in the Nextera DNA sample preparation guide (Illumina). The dual index strategy consists of incorporating unique indices into both ends of the library molecules to allow sample identification for the subsequent bioinformatics analysis (Kozich et al. 2013).

The 50 µl reaction mixture was made up of the following reagents: template DNA (40 ng), 1X Buffer HF, dNTPs (0.1 mM), Nextera index primers (index 1 and 2) and 1U Phusion DNA Polymerase system. The cycling parameters were those suggested by the Illumina Nextera protocol and are showed in Table 5.

The dual indexed amplicons obtained (336bp for V9 and 536bp for V4) were purified using magnetic beads AMPure XP (Agencourt Bioscience Corporation, Beverly, Massachusetts) at a concentration of 0.6X and 1X vol/vol (V4 and V9 amplicon, respectively), checked for quality control on Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California) and quantified by fluorimetry using the Quant-iTTM PicoGreen-dsDNA Assay Kit (Invitrogen, Carlsbad, California). The conditions of the second amplification are showed in Table 5.

2nd Amplification Conditions		
PCR Reaction	**DNA (amplicon)**	40 ng
	5X HF Buffer	1X
	dNTPs	f.c. 200 µM
	Illumina Nextera index 1 (N701…N712)	5 µl
	Illumina Nextera index2 (N501….N508)	5 µl
	P5 primer	f.c. 0.2 µM
	P7 primer	f.c. 0.2 µM
	Phusion DNA Polymerase	1.0 U
	H2O nuclease free	-
	Final volume	50 µl

	Step	**temperature**	**Time**
Thermal Cycles	**Initial Denaturation**	98°C	30 seconds
	5 cycles	98°C	10 seconds
		65°C	30 seconds
		72°C	3 minutes
	Hold	4-10°C	

Table 5. 2nd PCR Conditions

LIBRARY SEQUENCING

Equimolar ratios of the purified amplicons were pooled and subjected to paired-end sequencing on the MiSeq platform as reported below:

for V4 amplicon sequencing: dual indexed 2x250nt paired-end sequencing using MiSeq Reagent kit v3;

for V9 amplicon sequencing: dual indexed 2x150nt paired-end sequencing using MiSeq Reagent kit v2.

IMPORTANT NOTE

In order to increase the genetic diversity in the sequencing run, in our LifeWatch MoBiLab facility, in addition to the phage PhiX DNA library as required by the MiSeq, other genomic DNA libraries were added to the mix and co-sequenced.

The Index combination and number of sequenced reads obtained for each sample is shown in the Table 6.

			V9_18S_rRNA	V4_18S_rRNA
samples	N7--	N5--	N° paired-end	N° paired-end
2	N707	N502	838914	840992
3	N708	N502	610197	796011
9	N706	N504	599894	620305
14	N709	N502	859022	753794
22	N710	N502	754433	754220
30	N711	N503	757915	727139
37	N712	N503	772871	830003
39	N707	N503	727291	809799
43	N708	N503	848790	811252
49	N709	N504	650839	495287
54	N710	N504	796887	733874
55	N711	N504	614140	584611
60	N712	N504	775322	662592
71	N707	N505	948370	723665
72	N708	N505	1021305	843473
76	N709	N505	943112	574828
77	N710	N505	876288	719873
80	N711	N506	929506	837695
99	N712	N506	966666	819370
123	N707	N506	906272	749080
124	N708	N506	1036511	573516
132	N709	N507	899664	680953
141	N710	N507	846730	728238
143	N711	N507	889539	777685
146	N712	N507	964961	876863
149	N707	N508	731092	802958
150	N708	N508	813691	831386
152	N709	N508	889397	803915
4 sterivex	N710	N508	946274	723594
159	N712	N508	854119	802994
6	N701	N517	809996	
151	N702	N517	847547	

Table 6. Index combination and no. reads for V4 and V9 amplicon obtained for each sample