Interreg Alpine Space Eco-AlpsWater

Eco-AlpsWater

Innovative Ecological Assessment and Water Management Strategy for the Protection of Ecosystem Services in Alpine Lakes and Rivers

Priority 3: Liveable Alpine Space. SO3.2 - Enhance the protection, the conservation and the ecological connectivity of Alpine Space

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Illumina library preparation protocol: eDNA metabarcoding analyses of freshwater fish communities

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Interreg Alpine Space - Eco-AlpsWater project – WP1

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I. ABSTRACT

The aim of this document is to provide a detailed description of the Illumina library preparation protocol for eDNA metabarcoding analyses of freshwater fish communities, previously assessed and verified through an intercalibration test (Deliverable D.T1.1.2). This protocol will be used at the Sequencing and Genotyping Platform at FEM for the analysis of the samples collected in 2019 within the framework of EAW project.
The protocol in its present form, however, is still open to improvements and an additional protocol under testing by AGES will be applied to a selection of eDNA fish samples. This will allow to evaluate the replicability and robustness of approaches based on different protocols.

II. SAMPLE DESCRIPTION

The water samples have been collected by using an integrative sampling approach. This strategy is mainly based on two main principles: i) the collection of a large volume of water all along lake shorelines or all along the water columns (integrated depth sample) to be representative of the waterbody and to enhance the odds to collect rare DNA, and ii) a filtration in a closed cartridge (VigiDNA capsule filters) to gather eDNA occurring in the volume of water and to limit potential contaminations.
Moreover, in a selected number of lakes (i.e. Lake Bourget and Lake Garda), a point sampling approach has been used alongside the integrated approach in order to evaluate the benefits/limitations of both approaches. Point samples consist of ca. 2 L water samples collected at discrete locations, filtered using a Sterivex filter cartridge (0.45 µm) and preserved using the same buffer as the integrative samples. For robust statistical comparisons between the integrated and the point sampling approaches, it is recommended that for each integrated sample (i.e. each shoreline transect or each integrated depth sample) three discrete point samples are collected along the same transect or at the same locations for the shoreline and depth samples, respectively.

III. BRIEF OUTLINE OF THE LIBRARY PREPARATION APPROACH

Two different approaches are forecasted for the analysis of eDNA collected using Sterivex filters and VigiDNA filters: three PCR replicates for the amplification of Sterivex eDNA and nine PCR replicates for the amplification of VigiDNA eDNA will be performed, considering the higher volume filtered with VigiDNA filters (30 L vs 2 L) and the stochasticity of PCR method. A high number of replicates also allows to increase the probability to amplify very low amount of DNA templates, as it is expected for rare taxa. Therefore, this approach should guarantee the detection of rare native or invasive taxa occurring in low abundance. The PCR replicates will be used to prepare individual libraries which will be sequenced independently. This sequencing strategy allows to evaluate the detection variability among different replicates and to define the best approach to amplify the DNA present in trace amounts.

IV. MATERIAL CHECKLIST

IV.1 Primer details

Custom tagged primers for the first round of PCR

Custom tagged primers are used for the first round of PCR which represent the first step for the preparation of double tagged HTS libraries. The custom tagged primers were developed and validated using the EDITTAG software (Faircloth & Glenn, 2012) and consist of the MiSeq sequencing primers, a seven base-pair tag and the fish specific MiFish-U primers (Miya *et al.*, 2015). The complete sequences of six forward (F) and six reverse (R) custom tagged primers are given in Table 1. While it may be that not all primers will be used, it is highly

recommended to order all primers to have some back-ups in case some primer combinations will consistently fail to produce amplicons.

Table 1. List of six forward (F) and six reverse (R) custom tagged primers to be used during the first round of PCR amplification. The colour coded sequences represent the MiSeq sequencing primers (grey), the tags (blue) and the MiFish-U primers (green).

Tagged forward primers	
Primer ID	**Sequence (5'-3')**
MiFish-U-F-T113	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTACTACGTCGGTAAAACTCGTGCCAGC
MiFish-U-F-T161	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTAGAGTGTCGGTAAAACTCGTGCCAGC
MiFish-U-F-T097	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTTAGAGTCGGTAAAACTCGTGCCAGC
MiFish-U-F-T094	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTATCAGTCGGTAAAACTCGTGCCAGC
MiFish-U-F-T115	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTATGGAGTCGGTAAAACTCGTGCCAGC
MiFish-U-F-T118	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTCCATAGTCGGTAAAACTCGTGCCAGC

Tagged reverse primers	
Primer ID	**Sequence (5'-3')**
MiFish-U-R-T113	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTACTACCATAGTGGGGTATCTAATCCCAGTTTG
MiFish-U-R-T161	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTAGAGTCATAGTGGGGTATCTAATCCCAGTTTG
MiFish-U-R-T097	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTTAGACATAGTGGGGTATCTAATCCCAGTTTG
MiFish-U-R-T094	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTATCACATAGTGGGGTATCTAATCCCAGTTTG
MiFish-U-R-T115	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTATGGACATAGTGGGGTATCTAATCCCAGTTTG
MiFish-U-R-T118	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTCCATACATAGTGGGGTATCTAATCCCAGTTTG

Illumina tagged primers for the second round of PCR

During the second round of PCR, standard Illumina tagged primers are used to add the second set of tags and the MiSeq flow cell adaptors to the PCR product from the first round of amplification. Details of the Illumina tagged primers used during the second round of PCR amplification can be found in Table 2.

Table 2. Illumina tagged primers to be used during the second round of PCR amplification. The colour coded sequences represent the MiSeq flowcell adaptors (red) and a section of the MiSeq sequencing primers (grey). The inserts labeled as [i5] and [i7] represent the indices from the Nextera XT Index Kit A to D.

Primer ID	Sequence (5'-3')
P5 indexed primer	AATGATACGGCGACCACCGAGATCTACAC-[i5]-TCGTCGGCAGCGTC
P7 indexed primer	CAAGCAGAAGACGGCATACGAGAT-[i7]-GTCTCGTGGGCTCGG

Tagged primer strategy for the nine PCR replicates

For the amplification of the nine PCR replicates forecasted for VigiDNA eDNA analyses, a simple two-step tagging strategy can be used, allowing to limit the number of custom tagged primers in the first round of PCR (Table 3).

Table 3. Tagging strategy for the first and second round of PCR amplification to obtain nine independent PCR replicates by using only three couples of custom tagged primers in the first round. In the second PCR the tag *a* and *b* refers to the illumina P5 index and P7 index tagged primers.

Sample	PCR repl	1st PCR (tag)	2nd PCR (tag)
A	1	Tag 1	Tag a
A	2	Tag 1	Tag b
A	3	Tag 1	Tag c
A	4	Tag 2	Tag a
A	5	Tag 2	Tag b
A	6	Tag 2	Tag c
A	7	Tag 3	Tag a
A	8	Tag 3	Tag b
A	9	Tag 3	Tag c

IV.2 First round of PCR amplification

Instruments and other lab equipment and consumables

- ☐ PCR setup hood with UV-light
- ☐ Paper towel
- ☐ Latex gloves
- ☐ UV-sterilized 1.5 mL Eppendorf tubes
- ☐ Adjustable pipettes (0.2-2.0 µl, 2-20 µl, 20-200 µl)
- ☐ Aerosol barrier disposable tips for each pipette
- ☐ PCR tubes/plates
  The use of strip tubes with individual caps is recommended to avoid potential cross contamination.
- ☐ Tube racks
- ☐ Fine point permanent markers
- ☐ Mini vortex
- ☐ Mini (bubble) centrifuge
- ☐ Thermocycler for PCR reactions

- ☐ 10% bleach solution or other agent to remove DNA from workspaces (in spray bottle)
- ☐ 80% ethanol solution (in spray bottle)

Reagents

☐ Custom tagged MiFish-U primers	(10 µM)
☐ AmpliTaq Gold DNA Polymerase	(5 U/µL)
☐ GeneAmp Gold Buffer	(10 X)
☐ Magnesium Chloride ($MgCl_2$)	(25 mM)
☐ dNTP mix	(10 mM each)
☐ Bovine Serum Albumine (BSA)	(50 mg/mL)
☐ DNase/RNase free PCR H_2O	

IV.3 First pooling and clean-up

Instruments and other lab equipment

- ☐ Paper towel
- ☐ Latex gloves
- ☐ 1.5 mL Eppendorf tubes
- ☐ Pipettes (0.2-2.0 µl, 2-20 µl, 20-200 µl)
- ☐ Aerosol barrier tips for each pipette
- ☐ Tube racks
- ☐ Magnetic tube rack
- ☐ Fine point permanent markers
- ☐ Mini vortex
- ☐ Mini (bubble) centrifuge
- ☐ All instrumentation to run and visualize an agarose gel

Reagents

- ☐ AMPure XP beads
- ☐ 70% ethanol (**made fresh**)
- ☐ Elution buffer (H_2O, Tris-Acetate 10mM pH 8.0 or TE buffer)
- ☐ Reagents to prepare an agarose gel
- ☐ Gel loading dye
- ☐ 50 bp Ladder for gel electrophoresis

IV.4 Second round of PCR amplification

Instruments and other lab equipment

- ☐ As required for in-house protocols

Reagents

- ☐ As required for in-house protocols

IV.5 Final clean-up, normalization and pooling

Instruments and other lab equipment

- ☐ As required for in-house protocols

Reagents

- ☐ As required for in-house protocols

V. LIBRARY PREPARATION PROTOCOL

V.1 Notes to minimise contamination

- PCR reactions should ideally be set up in a room which is physically separated from any lab where fish DNA from tissue is extracted or PCR products are processed. If such a room is unavailable, the minimum requirement is that PCR reactions should be set-up in laminar flow hood which is only used for PCR set-ups and can be sterilized using UV-light. To thoroughly clean the flow hood, spray 10% bleach solution (or any other DNA removal agent) on a paper towel and wipe down the inside of the hood. Repeat the process with the 80% ethanol solution, close the hood and turn on the UV-light for 15 min before starting the protocol.
- All items to be used in the flow hood should not have been used elsewhere. If items such as pipettes etc. have been used outside of the hood for other protocols they should be thoroughly cleaned before starting the PCR set-up. To clean items, spray 10% bleach solution (or any other DNA removal agent) on a paper towel and wipe down all the items. Repeat the process with 80% ethanol solution and place items inside the flow hood before turning on the UV-light (see above).
- Clean other bench spaces that will be used during the process following the procedure described previously (i.e. wipe down with bleach and ethanol). Furthermore, it is highly recommended that at regular intervals all bench spaces will be cleaned using both bleach and ethanol.
- Always use gloves during the protocol steps. If gloves touch the inside of tubes/lids or any sample gets spilled on the gloves immediate change gloves.
- If samples get spilled on the work spaces or the pipettes, wipe down the area with bleach then ethanol and change gloves.

- Store all PCR reagents in a freezer which is exclusively used for the storage of reagents (i.e. no DNA should be stored together with reagents).

V.2 First round of PCR

During the first round of PCR, customized tagged primers will be used to amplify the MiFish-U barcoding region and simultaneously add the first set of tags to the amplicons. The primers required for this can be found in Table 1. Please note that each forward and reverse primer should only be combined so that in the resulting amplicon the forward and reverse tags are identical. The only allowed primer combinations for the first round of PCR are thus:

- MiFish-U-F-T113 & MiFish-U-R-T113
- MiFish-U-F-T161 & MiFish-U-R-T161
- MiFish-U-F-T097 & MiFish-U-R-T097
- MiFish-U-F-T094 & MiFish-U-R-T094
- MiFish-U-F-T115 & MiFish-U-R-T115
- MiFish-U-F-T118 & MiFish-U-R-T118

Before starting the protocol make sure to prepare a data sheet to determine how much mastermix to prepare and which primer combinations will be used for the different samples. An example data sheet is given with the details of the PCR mastermixes to prepare (Table 4) and the sample/PCR replicate layout for the PCR analyses (Table 5).

PCR set-up: stepwise protocol

1. Take all PCR reagents out of the freezer and leave them to thaw in a rack on the bench.
2. While waiting for the reagents to thaw, clean all bench spaces that will be used during the PCR setup following the protocol (see 'Notes to minimise contamination').
3. Next, set-up 3 x 1.5 mL Eppendorf tubes (for Sterivex eDNA samples) or 9 x 1.5 ml Eppendorf tubes (for VigiDNA eDNA samples) for the preparation of the mastermixes and label them MM1, MM2 and MM3… Place the tubes in a rack with the lids open.
4. Clean the PCR flow hood and any lab equipment that will be used in the hood (e.g. pipettes) following the previously described protocol (see 'Notes to minimise contamination'). Once all surfaces have been wiped down place all lab equipment in the hood together with the rack containing all the Eppendorf tubes (with lids open) and turn on the UV-light for 10 min.
5. When all reagents are thawed, briefly vortex all tubes to mix and spin down briefly in the mini benchtop centrifuge.

6. When the UV-sterilisation of the flow hood is finished transfer all reagents into the hood and prepare the PCR mastermixes by transferring the necessary reagents into the corresponding Eppendorf tubes (see table 4 for full details). PAY EXTRA ATTENTION WHEN ADDING THE COSTUMIZED TAGGED PRIMERS!!

Table 4. Example details of the PCR mastermixes for the analysis of Sterivex eDNA samples (3 PCR replicates). 8 samples and 10X reactions are considered (8 plus blank and 1 extra).

MasterMix (MM) label:		**MM1**	**MM2**	**MM3**
No. reactions:	**1X**	**10X**	**10X**	**10X**
Forward primer:	na	MiFish-U-F-T113	MiFish-U-F-T161	MiFish-U-F-T097
Reverse primer:	na	MiFish-U-R-T113	MiFish-U-R-T161	MiFish-U-R-T097
PCR H_2O	12.85 µL	128,5.00 µL	128,5.00 µL	128,5.00 µL
Gold Buffer	2.50 µL	25.00 µL	25.00 µL	25.00 µL
$MgCl_2$	2.00 µL	20.00 µL	20.00 µL	20.00 µL
BSA (50 mg/mL)	0.20 µL	2.00 µL	2.00 µL	2.00 µL
SYBR Green (5X)	0.60 µL	6.00 µL	6.00 µL	6.00 µL
dNTPs (10 mM)	0.65 µL	6.50 µL	6.50 µL	6.50 µL
Amplitaq Gold (5 U/µL)	0.20 µL	2.00 µL	2.00 µL	2.00 µL
Forward primer (10 µM)	1.00 µL	10.00 µL	10.00 µL	10.00 µL
Reverse primer (10 µM)	1.00 µL	10.00 µL	10.00 µL	10.00 µL
Environmental DNA	4.00 µL	na	na	na
Total volume:	**25.00 µL**	**250.00 µL**	**250.00 µL**	**250.00 µL**

7. Take out the appropriate number of optical PCR tube strips or a 96-well plate for PCR applications. Also, when using PCR tube strips with individual caps make sure to keep the tubes closed as much as possible during the procedure to avoid contamination.
8. Place tubes/plate into a rack and transfer the tubes/plate to the hood.
9. Briefly vortex the Eppendorf tubes containing the mastermix and spin down the tubes in the mini bench top centrifuge. DO NOT SPIN DOWN THE TUBES TOO LONG AS THIS WILL MAKE THE POLYMERASE SETTLE IN THE BOTTOM OF THE TUBES.
10. Prior to loading the mastermix in the PCR tubes/plate pipette up and down 10 time to ensure proper mixing of the mastermix. Afterwards, add 21 µL of the mastermix to the corresponding PCR tubes/wells (see Table 5 for the correct layout).
11. Once all required tubes/wells have been loaded with the correct mastermix, discard the Eppendorf tubes with the remaining mastermix and add 4 µL of PCR H_2O to the tubes/wells designated for the non-template controls (NTC) (see Table 5 for the correct layout when working with plates).
12. Remove the PCR tubes/plate from the hood and place them on the bench space to be used for the addition of eDNA extracts. Return all reagents to the freezer and repeat the cleaning process for the flow hood (see point 4 above).

Table 5. Layout of the samples and PCR replicates (rep) for PCR amplification. The layout follows the typical layout of a 96-well format with the colour codes of the wells indicating the mastermix to be used (see table 4).

	MiFish-U-F-T113 MiFish-U-R-T113	MiFish-U-F-T161 MiFish-U-R-T161	MiFish-U-F-T097 MiFish-U-R-T097	….		
	1	**2**	**3**	**4**	**5**	**6**
A	Sample1-rep1	Sample1-rep2	Sample1-rep3			
B	Sample2-rep1	Sample2-rep2	Sample2-rep3			
C						
D						
E						
F						
G						
H	NTC-rep1	NTC-rep2	NTC-rep3			

13. Take the eDNA extracts from the fridge/freezer and let them thaw if necessary. When fully thawed briefly vortex and spin down the eDNA extracts.
14. Load 4 µL of the eDNA extracts into the corresponding tubes/wells (see table 5 for the correct layout). Make sure to change pipetting tips between each tube/well to avoid the transfer of customized tagged primers from the mastermix to the eDNA sample and between the different tubes/wells.
15. When all eDNA samples have been added ensure that all PCR tubes are closed thoroughly or seal the 96-well plate.
16. Double check that all tubes of the eDNA samples are closed properly and return the eDNA samples to the freezer.
17. Clean the bench space used when loading the eDNA samples following the previously described protocol (see 'Notes to minimise contamination').

PCR run: stepwise protocol

1. Check again that the tubes/plate have/has been sealed properly to avoid evaporation and briefly spin down the PCR tubes/plate.
2. Place the PCR tubes/plate on the PCR instrument making sure that tube/well positions correspond to the outline provided in Table 5.
3. Set-up the correct cycling conditions (Table 6):

Table 6. Thermal cycling conditions for the PCR set-up used during the first round of PCR amplification.

Description	Temperature (°C)	Time (min:sec)	No. cycles
Activation	95	05:00	1
Cycling	95	00:30	35
	65	00:30	
	72	01:00	
Extension	72	10:00	1
Hold	10	∞	

4. Start the run and verify that everything is running properly before leaving.
5. Check on a 2% agarose gel the PCR amplification product to verify that for each of the mastermixes at least one of the samples showed positive amplification. If this is not the case it is likely that the combination of the custom tagged primes used in this mastermix does not work optimally and an additional run will need to be performed using a different set of primer combinations.
6. Store tubes/plate with the amplicon products at 4°C or immediately proceed to the first pooling and the clean-up step.

V.3 Pooling and clean-up of first PCR products

During this step we pool the amplicons generated from each sample and clean-up the products using a left-handed size selection protocol. The obtained product will then be diluted and used as template in the second round of PCR amplification. THROUGHOUT THIS PROTOCOL IT IS HIGHLY RECOMMENDED TO KEEP ALL SAMPLES AND INTERMEDIATE PRODUCTS UNTIL SEQUENCING HAS CONFIRMED THE CORRECT PROCESSING.

Pooling: stepwise protocol

1. Clean all bench spaces and lab equipment (i.e. pipettes) following the previously described protocol (see 'Notes to minimise contamination')
2. Briefly spin down the tubes/plate with the amplicon products from the first amplification round to ensure all products are at bottom of the tubes/wells.
3. Set-up 1.5 ml Eppendorf tubes labelled with the sample ID's.
4. In the first set of labelled Eppendorf tubes transfer 15 µL of PCR product obtained from each PCR replicate of the respective sample (see Table 7). PRIOR TO TRANSFERRING ALIQUOTS PIPET UP AND DOWN THREE TIMES TO MIX.

5.

Table 7. Schematic overview of the first pooling step where the PCR product obtained from the different PCR replicates of a single sample will be combined.

	Amplicons from 1st PCR round				1st Set		2nd Set
	1	2	3				
A	Sample1-rep1	Sample1-rep2	Sample1-rep3	15 µL each	**Sample1**	5 µL	**Sample1**
B	Sample2-rep1	Sample2-rep2	Sample2-rep3		**Sample2**		**Sample2**
C							
D							
E							
F							
G							
H	NTC-rep1	NTC-rep2	NTC-rep3	15 µL each	**NTC**	5 µL	**NTC**

6. Once the products of the PCR replicates for each sample have been pooled, transfer 5 µL of the pooled product into the second set of Eppendorf tubes. Store the 5 µL aliquots at 4°C (these will be used later to check the performance of the clean-up protocol).

Clean-up: stepwise protocol

1. Set up a three additional series of Eppendorf tubes labelled with:
    a. Series 3 & 4: the sample ID's followed by 'CLEAN'.
    b. Series 5: the sample ID's followed by 'CLEAN 1:10'.

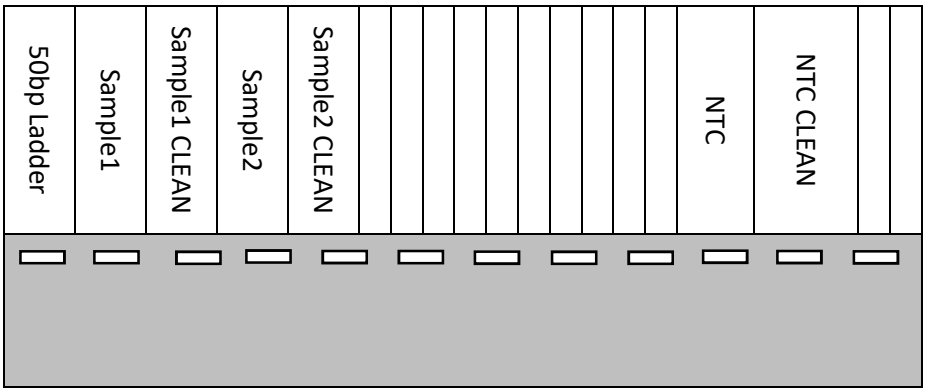
   These tubes will be used to collect the purified PCR products and make up the dilution for further use at the end of the protocol (see below).
2. Bring the AMPure XP beads to room temperature and briefly vortex them to make sure the beads are evenly dispersed.
3. Briefly vortex and spin down all the tubes containing 40 µL of the PCR product.
4. Add 40 µL of AMPure XP beads to each Eppendorf tube (i.e. 1:1 volume ratio of beads vs PCR product) and gently pipette up and down 10 times to ensure proper mixing.
5. Incubate at room temperature for 5 min.
6. Place the tubes in a magnetic rack for 2 min or until the supernatant has cleared.
7. Gently aspirate and discard the supernatant from the tubes without disturbing the pellet.
8. Perform two washing steps following the below process:
    a. Add 200 µL of freshly prepared 70% ethanol to each tube and incubate for 30 sec.
    b. Gently aspirate and discard the supernatant without disturbing the pellet.
9. After the two washing steps allow the beads to air-dry for 5-10 min.
10. Remove the tubes from the magnetic rack and add 40 µL of elution buffer (H_2O, Tris-Acetate (10mM pH 8.0) or TE buffer).

11. Gently pipette up and down to mix the beads with the elution buffer. Make sure the beads are fully resuspended in the elution buffer.
12. Incubate at room temperature for 5 min.
13. Place the tubes back on the magnetic rack for 2 min or until the supernatant has cleared.
14. Transfer the supernatant to the third set of tubes prepared above. Additionally, transfer 5 µL of the cleaned amplicons to the fourth set of tubes and store these at 4°C.
15. In the fifth set of Eppendorf tubes make up a 1:10 dilution of the cleaned up PCR amplicons (i.e. 10 µL of cleaned product + 90 µL H_2O).
16. Store all tubes at 4°C.

Visualize amplicons before and after the clean-up protocol

1. Prepare a 2% agarose gel with proper number of wells.
2. Take out the 5 µL aliquots of the amplicon pools taken before and after performing the first clean-up step. Briefly spin down the samples to ensure all product is at the bottom of the tubes.
3. Add 1 µL of gel loading dye to each tube and briefly spin down again.
4. Load the samples (5 µL) and a 50 bp Ladder (3 µL) on the gel following the layout specified in Table 8.
5. Run the gel at 120 V for 30 min and visualize using a gel imagining system (amplicons are expected to be around 350-400bp long).

Table 8. Layout to use when loading the samples on the agarose gel.



V.4 Second round of PCR

The second round of PCR is used to add dual Illumina indices and the Illumina sequencing adapters to the amplicons generated from during the first round of PCR. This step can be performed according to the in-house protocols.

An aliquot of the 1:10 diluted and cleaned up PCR product should be used as template DNA during the second round of PCR with 8-10 amplification cycles. A second round of PCR should be conducted for all samples (i.e. eDNA samples and NTC) even if during first round of PCR no bands were observed.

V.5 Final clean-up, normalization and pooling

The final clean-up of the sequencing libraries is performed using the AMPure XP beads with a 1:1 volume ration of beads vs PCR product.

Once all libraries are cleaned, they should be quantified (recommended to use fluorometric quantification using dsDNA binding dyes) and normalized to 4 nM. To determine the DNA concentrations in nM while taking into consideration the size of the amplicons the below formula can be used:

$$DNA\ conc\ (nM) = \frac{\left(DNA\ conc\ (ng/\mu L)\right)}{(660\ g/mol\ \times amplicon\ size)} \times 10^6$$

For the libraries generated based on this protocol the average amplicon size should be around 375 bp. Once DNA concentrations are calculated, normalize the libraries and pool equal volumes of the normalized libraries for sequencing.

IMPORTANT: The final DNA concentration of negative controls may fall below the 4 nM. Nonetheless, aliquots of the samples will need to be included in the final pooling as it would allow us to determine possible contamination rates. For these samples, use the standard volume of the undiluted PCR product during the final pooling step.