eDNA Expeditions
eDNA sampling protocol

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BACKGROUND

This document describes the required protocol to conduct environmental DNA (eDNA) sampling as a part of UNESCO eDNA Expeditions and using the citizen science sampling kits.

See also the:

- UNESCO eDNA Expeditions field sampling booklet, instruction video, infographic and sample information sheet
- Sylphium eDNA kit instructions
- UNESCO eDNA expeditions sample registration application

SUMMARY

UNESCO eDNA Expeditions is a global, citizen science initiative that will help measure marine biodiversity, and the impacts climate change might have on the distribution patterns of marine life, across UNESCO World Heritage marine sites. It is a citizen science initiative, whose objective is to engage local communities in sampling for eDNA and inspire the next generation of ocean professionals. Water sampling is based on citizen science sampling kits, which make sampling easy and with minimal required materials and training.

Water samples are taken with syringe and filtered through a dual filter capsule of 0.8 µm pore size. It is recommended to filter at least 1.5 liters of water (25x 60 ml syringe). After filtering the filter capsule is emptied of water. Then Longmire's solution is added to the filter to preserve the DNA. A salinity sample and a temperature reading are also taken. The filter, salinity sample, and metadata sheet (i.e., sample sheet) are returned to the UNESCO team for processing.

METHOD DESCRIPTION AND RATIONALE

This method was chosen as it is easy to execute and possible to perform in remote areas, without access to equipment or specific materials. Longmire's solution was chosen as a preservation liquid due to it being non-toxic. Sampling is being conducted by different groups of citizens, and especially youth groups.

SPATIAL COVERAGE AND ENVIRONMENT(S) OF RELEVANCE

eDNA sampling campaigns were conducted at 21 UNESCO World Heritage marine sites. During the first campaign, 21 sampling kits were sent to each

site. Five sampling locations were selected by the local site authority to represent different habitats or to target known locations of specific species. At each site, four replicate samples were collected, spaced approximately 5-10 meters apart. The site had to have a total water depth of less than 15 meters, as sampling was done in surface waters. The scientific advisory board determined that relevant detections could not be made in areas with deeper water columns using the current method.

PERSONNEL REQUIRED

A minimum amount of 3 people is required to conduct sampling comfortably. One to retrieve the sample and perform the filtering, one to support the sample bag and ensure that all steps of the sampling instructions are followed closely, and one to record metadata during the sampling on the sample sheet and in the app.

SAFETY

Sampling should only be performed in favorable weather conditions, and care should always be taken when performing sampling for the safety of the people sampling.

TRAINING REQUIREMENTS

The people sampling should familiarize themselves by carefully reading through the sampling protocol and viewing the sampling video prior to the sampling campaigns. Other training is not required. The sampling protocol should be consulted throughout the sampling, and is available in the booklet, on the sampling sheet and in the application.

TIME NEEDED TO EXECUTE THE PROCEDURE

At least 30 minutes are required for one sample.

EQUIPMENT

DESCRIPTION	PRODUCT NAME AND MODEL	MANUFACTURER	QUANTITY	REMARK
DURABLE EQUIPMENT				
Sample bottle	Angular beaker 1000 ml	Burkle	1	The bottle and rod are used to collect surface water at the site, any alternative methods are ok
Sample rod	Telescopic rod 95-280 cm	Burkle	1	See remark above
Bucket for support	ø146 mm 1500 ml (TE) plastic bakje met deksel	Intopack BV bought through https://packstore.nl/	1	This is used to support the stand-up 2 liter sample bag. Any container of the right dimensions will work
Thermometer	Digital aquarium thermometer	Bought through https://reefaquariumstore.com/	2	We have had issues with these and the battery endurance, alternative methods for collecting temperature data are recommended
Safety glasses	Sur-lunettes de protection 3M Visitor	3M bought through Lyreco	4	Any alternative ok
CONSUMABLE EQUIPMENT				
eDNA filter and valve	eDNA Dual Filter™ PES 0.8 µm pore size and dual check valve	Sylphium	1	Sterile
60 ml syringe	50 ml BD Luer-Lok™ sterile syringe	BD Plastipak	1	Sterile
5 ml syringe	5 ml Norm-Ject® sterile syringe	B.Braun	1	Sterile, for the preservation liquid
Luer-lock cap	Male-female long cone Luer cap	Muroplas	2	Sterile, to close the filter after filtering. One of these used to close the 15 ml syringe with the preservation liquid before use.
Sample bag	Stand-up 2 litre sample bag	Nasco Whirl-Pak	1	Sterile, used to collect the sample water in for filtering.

DESCRIPTION	PRODUCT NAME AND MODEL	MANUFACTURER	QUANTITY	REMARK
Zip-lock bag	150 x 220 mm PE transparant, 50µ	Boom Meppel	1	This is to return the filter in a separate bag to the rest of the material.
Salinity tube	15 ml falcon tube	Sarstedt	1	To collect a sample of water for salinity measurements.
Gloves	econitril PF 250 Gloves (pair) size M	SHIELD	1	To enable clean sampling.
Sample-ID stickers	Waterproof labels with sample IDs		3	To label the filter, the salinity sample and the sample sheet with the unique sample IDs.
Bottled water	1.5 litres of drinking water		1.5 L	Used as a negative control for the sampling conditions.
CHEMICALS				
Preservation liquid	Longmire's buffer	Sylphium	3.5 ml	Non-toxic preservation liquid, can precipitate in cold conditions
Tris	Cat. No. T1501.1000	Duchefa	100 mM	Final concentration in preservation liquid
EDTA	Cat. No. 147850010	Thermo scientific	100 mM	Final concentration in preservation liquid
NaCl	Cat. No. S0520.1000	Duchefa	10 mM	Final concentration in preservation liquid
SDS	Cat. No. 11667289001	Roche	0.5 %	Final concentration in preservation liquid

STANDARD OPERATING PROCEDURE (SOP)

In the following SOP, please use the exact names of equipment as noted in the table above.

PROTOCOL

SAMPLING

The goal is to collect 21 samples from each participating UNESCO World Heritage marine site, including one negative control sample (local bottled water) at the end of each sampling campaign.

- Each UNESCO World Heritage marine site has determined 5 sampling locations before the start of the sampling day.
- Take 4 replicate samples at each location (same time and location). It is recommended to take replicate samples within 5-10 meters of each other, but this can vary depending on the size of the habitat being sampled.
- The total water depth of the sampling locations should be shallower than 15 meters (to ensure that the collected eDNA is representative for the sample location).
- Each sampling will provide a snapshot of marine biodiversity. The objective is to take all samples during one day across the UNESCO World Heritage marine site. If this is not possible, there is flexibility to choose two different dates for sampling.

  Warning: Avoid contamination sources, e.g. all human activity, including the bilge pumps of vessels, fish cleaning stations, or port areas.

PREPARATION

Please specify the preparatory actions you took before you collected the samples and note what equipment was needed to do so (e.g. disinfection of work surfaces, preparations to the equipment you intend to use later on).

- **Step 1: Determine 5 sampling locations**

Determine 5 sampling locations before the start of the sampling day. The sampling locations should be shallower than 15 meters (to ensure that the collected eDNA is representative of the sample location).

- **Step 2: Download the sample registration application**

Download the sample registration application. The application will automatically collect time and location information in the field and allows metadata to be registered and linked to each sample-ID.

1. Go to the address https://app.ednaexpeditions.org/ on your mobile phone.

2. Add the application to the home screen. Allowing this will show the application as a normal application on the phone.

3. Allow location services for the application, to register the exact location where samples were taken.

All information on the application is strictly used in the context of this project.

4. Register with your email, and you are ready to go!

5. To start sample registration, press: Add sample.

Once downloaded, the sample registration application will also work without internet access. After recording the sample information, press save. The information will be automatically uploaded once the mobile phone has access to the internet again. To ensure that all information is uploaded after sampling, make sure to open the sample registration application at least once after your internet connection has been restored. The application includes an electronic version of this Field Sampling Booklet, and the video with sampling instructions (internet access required).

- **Step 3: Bring with you the following materials**

Provided materials:

1. Sample beaker and rod

2. Thermometer; Check that it is functioning

3. Small container to support the sample bag

4. Field sampling booklet

5. Safety glasses

6. 21 sampling kits and 21 sample information sheets (inside)

7. 21 pencils and 4 clipboards

Materials that need to be provided yourself:

1. 1.5 liters of bottled (clean) water to filter for the negative control

2. A box or cooler to store the filters away from direct sunlight after sampling

3. A mobile phone with the sample registration application installed

- **Step 4: Double check the completeness of the sampling kit**

Double check the completeness of the sampling kit and the unique sample ID of your filter. Go through the instructions and make sure you understand what needs to be done to filter the sample. In each sampling kit you will find:

1. The sample information sheet
2. Two pairs of gloves
3. A clean and sterile sample bag
4. A sterile 60 ml syringe
5. The eDNA filter with the valve
6. 2 blue caps to close the filter
7. The preservation liquid in a small syringe
8. Three waterproof labels with the unique sample ID
9. A zip-lock bag for the filter
10. A 15 ml tube to collect a sample for salinity

SAMPLING

- **Step 1: Initiate sampling**

1. When you have arrived at the sampling location, open the sample registration application and take the sample information sheet. Sample information should be recorded twice, once in writing on the sheet, and a second time electronically in the application.

- Write down the name and email address of the person(s) sampling.
- Record the sample ID (the code located on three labels inside the kit, e.g. “EE0018”).
- Record the sampling time and location by tapping on the application and write down the same information on the sample information sheet.
- Also record information about the sampling site: what is the biome (e.g., seagrass beds, coral reefs, kelp forest, sand, mud, rock, mussel

beds, mangrove forest, estuary)? Are there any visible blooms of algae, or any other organisms?
- Remember you can also add photos and sampling selfies on the application. This will be helpful in determining what the sampling conditions were, and contribute to the project's global communication.

- **Step 2: Collect environmental metadata**

2. To determine the salinity, collect 15 ml of water from the sampling location in the separately provided 15 ml tube, then return the tube to the sample kit. Knowing the salinity will improve the final analysis of the samples, since low salinity can reveal runoff from land, for example.

3. Record the surface water temperature at the sampling location with the use of the provided thermometer. Add this information in the sample registration application, and write it down on the sample information sheet.

FILTRATION

Wear gloves at all times during sampling. Do not touch anything other than the sampling equipment to avoid contamination. Also, keep the sampling equipment as clean as possible, and store it in a clean place.

- **Step 1: Collect the water**

4. Rinse the sampling bottle with the rod three times with water from the sampling location.

- If you are close to the shore, aim to take samples as far away from land, and you, as possible (using the rod), to avoid contamination (such as runoff from land, or human presence in the water).

5. Collect the sample, make sure to immerse the bottle approximately 30 cm below the surface.

6. Prepare the sample bag: Rip open the top and open the bag using the holders on the sides.

7. Place the sample bag inside an empty plastic box (for support).

8. Fill the sample bag two times with water collected with the bottle, in total about 2 liters of water. One person should hold the sample bag, while the other does the filtering.

Note: If you are in an area with a sandy bottom, let the sample water stand for a few minutes before filtering, to allow the sand to settle at the bottom of the bag.

- **Step 2: Filter the water**

9. Attach the syringe to the valve on the filter.

10. Be careful, proceed slowly, the next step will require some effort and patience. If needed, take turns with the person holding the bag.

11. While the inlet (bottom part) of the filter is submerged in the sample water, filter the water from the bag by pulling out the plunger. The water will flow in the direction of the arrow on the filter.

12. Discard the water outside of the bag by pushing on the plunger. The water will come out of the other end of the valve.

13. Count the number of times the syringe is filled and/or the amount of water that you have filtered. Add this information both in the sample registration application and on the sample information sheet.

14. When the filter is full, the filtering will be slower. To keep filtering, pull out the plunger of the syringe and allow the water to flow in slowly. Depending on the locality, there may or may not be color visible on the filter.

15. Aim to repeat 20-30 times (25 times = 1.5 liters) by continuously pumping the syringe and keeping the (bottom part of the) filter submerged. The final amount of water that can be filtered will depend on local conditions. Stop if the filtering is too slow or is taking too long (e.g. one hour). The filter is then clogged.

Warning: Avoid air bubbles in the filter, as they can cause the filter to malfunction. To prevent air from being drawn in, release the plunger of the syringe before lifting the dual filter capsule from the water. If air bubbles appear in the filter during the filtering process, they can be easily removed. For example, if filtering becomes difficult after fewer than 10 cycles, an air bubble may be clogging the filter. In this case, the air bubble can be removed easily—see steps 25-30.

SAMPLE PRESERVATION

Perform these steps once you have filtered the volume of the syringe 20-30 times (25 times = 1.5 liters = 1500 ml) or for about one hour. This step should be performed by an adult wearing gloves and safety glasses.

- **Step 1: Empty the filter**

16. Empty the remaining water from the filter by sucking in air with the syringe.

17. Remove the filter from the syringe.

- **Step 2: Add the preservation liquid**

18. Wear the safety glasses, be very careful with the preservation liquid, do not swallow, avoid contact with skin and eyes, and do not spill in the environment. All preservation liquid should go into the filter, no preservation liquid should be left.

Note: If there are white flocs in the preservation liquid, warm it by holding the syringe in your hand, until they dissolve. The white flocs can occur if the preservation liquid has been exposed to temperatures below and around +15 °C.

19. Close the top end of the filter with the cap from the small syringe. The top end is where the valve and filter were. The flow of the preservation liquid will be in the same direction as the sample water during filtering.

20. From the bottom end of the filter (in the direction of the arrow), add the preservation liquid to the filter from the provided small syringe.

Warning: When adding liquid, pressure builds up in the filter. Relieve the pressure by releasing the plunger before removing the syringe, allowing air from the filter to replace the liquid in the small syringe.

21. Close the bottom end of the filter with the second cap provided in the sampling kit.

22. Attach the three labels provided in the sampling kit to

a. the filter,

b. the salinity sample, and

c. the sample information sheet.

Make sure that the same sample ID is registered on the sample registration application.

23. Close the filter in its own zip-lock bag. This will allow the filter to stay clean also when starting sample processing.

24. Enclose all materials including the filter (in its own bag), the salinity sample, and the sample information sheet in the original sample bag. Save the sample information collected in the application.

STORAGE

Keep the filters sheltered from the sun at all times, as UV radiation can degrade the collected DNA. All samples should be collected with the sampling team, kept in the dark, and stored at room temperature (e.g., 20-25°C) if possible. The most important thing is to keep the filters away from direct sunlight. Some heat will not negatively impact the filters or the DNA.

QUALITY CONTROL

A negative control is filtered to identify common contaminants of the sampling process. This is 1.5 liters of bottled (drinking) water.

- After all marine samples have been filtered, filter the negative control (1.5 liters of bottled water) while still in the field using the exact same protocol.
- Start by recording the sample ID and indicate on the sample information sheet and in the notes in the sample registration application that this is the negative control. There is no need to collect environmental metadata (location, time, etc.).
- Pour the bottled water in the sample bag and proceed with filtering and preserving the sample as before.

BASIC TROUBLESHOOTING GUIDE

REMOVING AN AIR BUBBLE

Only perform steps 25 to 30 if you suspect you have an air bubble clogging the filter. Air bubbles block the filter membrane and have a negative effect

on the filter capacity. These air bubbles are easy to remove from the capsule, by flushing a small amount of sample water through the outlet of the filter (reverse direction).

25. Remove the filter from the syringe, and the valve from the filter from the silicone tubing connected to the valve.

26. Fill the syringe with sample water.

27. Connect the syringe to the outlet of the eDNA dual filter capsule.

28. Turn the filter upwards, so that any air bubble moves up to the inlet.

29. Hold the capsule upwards and push the syringe plunger until all air bubbles have been expelled from the capsule.

Warning: Do not allow water to be flushed out of the filter, this will result in the loss of already filtered material.

30. Reconnect the capsule and syringe to the valve connector and proceed with sampling.

NOTE: SAMPLING FROM SHORE

Avoid contamination especially if it is necessary to sample from shore.

- Only one person should go briefly in the water to collect the sample.
- Extend the rod as far as possible to take the sample from a distance to yourself.
- Consider the flow of water at the sampling location:
    - Try to take the sample so the water is flowing towards the person that is sampling (not from the person towards the sampling location).
    - E.g. take the sample during incoming tidal flow, or to the side of the person taking the sample
- If available, it is always best to wear waders (if possible disinfected with bleach).

NOTE: PRESERVATION LIQUID

Care should be taken, especially during the step of adding the preservation liquid. Because the preservation liquid is added to a closed filter, some

pressure builds up inside. It is crucial that the preservation liquid is added from the correct end (the bottom end) of the filter, ensuring that the liquid flows in the same direction as the sampled water, as indicated by the arrow. After adding the preservation liquid, some air should be drawn out with the syringe before removing the small syringe, to prevent any preservation liquid from spilling out of the filter due to the pressure buildup.

FAILURE OF THE TWO-WAY VALVE

We noticed that some of the two-way valves provided in the sampling kits were leaking air, making them less effective. In this case, the extra cap can be used to seal the valve's outlet when filtering water through the filter and the valve's inlet. This will close the system and result in more effective filtering.

eDNA Expeditions DNA extraction following the Sylphium protocol

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BACKGROUND

This document describes the required protocol to conduct DNA extraction of dual filters as a part of UNESCO Environmental DNA (eDNA) Expeditions. This protocol is based on the protocol for Sylphium eDNA kits.

SUMMARY

UNESCO eDNA Expeditions is a global, citizen science initiative that will help measure marine biodiversity, and the impacts climate change might have on the distribution patterns of marine life, across UNESCO World Heritage marine sites. It is a citizen science initiative, whose objective is to engage local communities in sampling for eDNA and inspire the next generation of ocean professionals. Water sampling is based on citizen science sampling kits, which make sampling easy and with minimal required materials and training.

This protocol explains the DNA extraction procedure from the dual filters. This protocol is a copy from the Sylphium DNA extraction protocol.

METHOD DESCRIPTION AND RATIONALE

DNA extraction from the eDNA dual filters was performed using the eDNA isolation kits available from Sylphium.

The Sylphium Environmental DNA Isolation Kit procedure is suitable for qualitative and quantitative PCR amplification.

SPATIAL COVERAGE AND ENVIRONMENT(S) OF RELEVANCE

eDNA sampling campaigns were performed across 21 UNESCO World Heritage marine sites across 19 countries. All samples were treated the same for DNA extraction and PCR.

PERSONNEL REQUIRED

Highly trained and skilled molecular laboratory scientists/technicians are required to conduct this work.

SAFETY

Working with DNA and in the molecular laboratory requires high awareness of safety and risks associated with the chemicals. In addition, it is very

important to work in a clean space and avoid contamination of samples at any point in the sample processing. See guidelines for clean eDNA work.

TRAINING REQUIREMENTS

A specialized training (usually university level) for the molecular laboratory is required for this work.

TIME NEEDED TO EXECUTE THE PROCEDURE

The time needed is highly dependent on the number of samples being processed. The whole procedure for isolation of total eDNA takes about 2 hours. The isolated DNA will be dissolved in TE and is ready to use in qPCR.

EQUIPMENT

DURABLE EQUIPMENT

- Pipettes
- Microcentrifuge (13,000 x g)
- -20 ° freezer
- Centrifuge for 15 ml tubes
- Incubator or heat block at 55 °C
- Tecan plate reader

CONSUMABLE EQUIPMENT

- Sylphium eDNA isolation kit
- Pipette tips
- Ice
- 15 ml tubes with cap
- 2 ml tubes with lit
- 96 wells qPCR plates + seal cover
- PCR cooling rack (optional)
- 96 wells qPCR plates + seal cover

CHEMICALS (ALL PROVIDED WITH THE SYLPHIUM EDNA EXTRACTION KIT)

- S1 (optional): Buffer with internal negative control
- S2: Phase separation solution
- S3: Precipitation buffer
- S4: Precipitation solution

- S5: Wash solution
- S6: eDNA conservation buffer

Store reagents and kit components for DNA isolation at room temperature (15-30°C).

STANDARD OPERATING PROCEDURE

PROTOCOL

PREPARATION

All work is to be performed in a clean room specifically prepared for the processing of eDNA samples to avoid all possible sources of contamination.

DNA EXTRACTION FROM WATER FILTERS

1. (Not performed but possible) **Incubate the eDNA dual filter capsule containing the preservation liquid at 55 - 60 °C for 60 min to release all DNA from cell material**. Samples older than one day are already lysed at room temperature and can be used directly in step 4.

2. **Prepare 15 ml tubes with 750 µl S2 (Phase separation solution)**. Prepare as many tubes as the amount of samples present, plus one additional tube. This additional tube will be the internal negative control

(INC). Preparing these tubes before handling the samples will prevent contamination of liquids present in the kit.

3. **Prepare 2 ml tubes with 100 µl S3(Precipitation buffer) and 900 µl S4 (Precipitation solution)**. Prepare as many tubes as the amount of samples present, plus one additional tube. This additional tube will be the INC. Preparing these tubes before handling the samples will prevent contamination of liquids present in the kit.

4. **Remove the cap of the outlet of the filter capsule and connect the 60 ml syringe (used during sampling) on the outlet**.

5. **Keep the filter capsule above the syringe, remove the second cap and pull the plunger slowly until the liquid is inside the syringe**. Take care not to spill any of the DNA containing liquid. This can be a source of DNA contamination for other samples. If any spill occurs, clean all contaminated material with DNA decontamination reagent.

6. **To maximize liquid extraction from the filter capsule, push and pull the plunger slowly a few times (air in the capsule, liquid out)**. Be aware not to push the liquid back into the filter capsule.

7. **Add the liquid inside the syringe to the 15 ml tube containing 750 µl S2 (Phase separation solution)**. Number this tube with the number written on the sample bag.

8. **Take the additional 15 ml tube and add 1 ml S1 (INC) into this tube, mix by shaking and mark this tube as INC**. This is the INC and it should be handled in the coming steps like the other samples.

9. **Centrifuge samples at +/- 4.000 x g for 5 minutes**. A bilayer will be formed. The top layer is the water phase and contains the DNA. Between the two liquid layers a small solid layer with denaturated proteins can be formed.

10. **Transfer max 1000 µl of the water layer to the prepared mixture (S3 and S4) and mix well by gently shaking and cool down to -20 °C for at least 20 minutes**. In this step DNA will precipitate while salts and other unwanted substances will stay in solution. Prevent transfer of the protein and CIA layer to the new tube. If a metal cooling block is placed in the freezer, this step can be shorter.

11. **Transfer the remaining water layer (+/- 1000 µl) to an empty 2 ml tube and use this as a backup**. Store this tube in a -20 °C freezer. If no backup is required, these samples can be used in extraction to maximize eDNA recovery from the sample. Make additional tubes as described in step 2 if these samples will be isolated as well.

12. **Centrifuge samples for 20 minutes at maximum speed (11.000 g)**. Remove supernatant by decanting and pipetting. Be careful, the DNA pellet is not firmly attached to the tube. It is important to remove all liquid to increase sensitivity of qPCR detecting.

13. **Add 500 µl S5 (Wash solution), mix and centrifuge samples at 11.000 g for 5 minutes. Remove supernatant completely by pipetting and remove all liquid. Repeat this step to remove PCR inhibiting substances**. Be careful, the DNA pellet is still not firmly attached to the tube.

14. **Dissolve the pellet in 100 µl S6 (eDNA conservation buffer) and use 2 µl for qPCR**.

QUALITY CONTROL

Concentration was measured with the Quant-iT PicoGreen dsDNA kit (Invitrogen, Life Technologies) according to the manufacture's guidelines.

Step 1: Prepare 1 x TE buffer

- 200 µl needed per sample.
- For 100 samples prepare 20 ml 1xTE buffer:
  - 1 ml 20xTE buffer
  - 19 ml high pure water
- Store in falcon tube at RT (long-term storage).

Step 2: Prepare standard dilution series

- Label 6 eppendorf tubes.
- Pipet 1xTE buffer first, no need to change filter tip.
- Mix by pipetting:
  - Tube 1 = dilution (1/100; 1 ng/µl) = 198 µl 1x TE + 2 µl of Lambda stock DNA (100 ng/µl)
  - Tube 2 = dilution (1/2; 0.5 ng/µl) = 100 µl 1xTE + 100 µl tube 1
  - Tube 3 = dilution (1/2; 0.25 ng/µl) = 100 µl 1xTE + 100 µl tube 2
  - Tube 4 = dilution (1/5; 0.05 ng/µl) = 160 µl 1xTE + 40 µl tube 3
  - Tube 5 = dilution (1/5; 0.01 ng/µl) = 160 µl 1xTE + 40 µl tube 4
  - Tube 6 = Blank (1) = 100 µl 1xTE
- Pipet 100 µl of each tube into Greiner plate.

Step 3: Prepare samples

- Pipet 99 µl 1xTE buffer (prepared in step 1) into Greiner plate.
- Add 1 µl DNA sample (= 1/100 dilution).

Step 4: Prepare Quant-iT reagent

- Note: Prepare reagent just before use!
- 100µl needed per sample and standard.
- For 100 samples prepare 10 ml Quant-iT reagent:
  - 50 µl 200x Quant-iT reagent (blue cap)
  - 9950 µl 1xTE buffer (prepared in step 1)

Step 5: Add Quant-iT reagent to samples and standard dilution series

- Add 100 µl Quant-iT reagent to each well of the Greiner plate (samples and standards) with multipipet.
- Wrap plate in aluminium foil and measure within 5 – 10 minutes.
- Measure with Tecan plate reader.
- Calculate concentrations.

eDNA Expeditions PCR protocol

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BACKGROUND

This document describes the required protocol to conduct amplification of target genes as a part of UNESCO environmental DNA (eDNA) Expeditions.

SUMMARY

UNESCO eDNA Expeditions is a global, citizen science initiative that will help measure marine biodiversity, and the impacts climate change might have on the distribution patterns of marine life, across UNESCO World Heritage marine sites. It is a citizen science initiative, whose objective is to engage local communities in sampling for eDNA and inspire the next generation of ocean professionals. Water sampling is based on citizen science sampling kits, which make sampling easy and with minimal required materials and training.

This protocol explains the amplification of target biomarkers from the UNESCO eDNA Expeditions project. The objectives of the project were to target fish and megavertebrates, and the benefit of citizen science engagement for this work.

METHOD DESCRIPTION AND RATIONALE

The project has the broad goal of testing eDNA sampling for the description of fish and vertebrate biodiversity at the sites sampled by citizen scientists. To this end, multiple biomarkers were chosen to ensure a large target diversity. While most markers were targeting fish, mammal targets were also included as well as a broad-range CO1 target biomarker to give insight to biodiversity at the sites at large, compare across the vastly different sites and have the possibility of detecting invasive or other rare target species.

Eight different biomarkers were chosen for the analysis of biodiversity from the sites.

Primer Set	Loci	F-primer	R-primer	Length	Reference
Mifish-U	12S	GTCGGTAAAACTCGTGCCAGC	CATAGTGGGGTATCTAATCCCAGTTTG	171	Miya et al. 2015
Mifish-E	12S	GTTGGTAAATCTCGTGCCAGC	CATAGTGGGGTATCTAATCCTAGTTTG	171	Miya et al. 2015
Mimammal-U	12S	GGGTTGGTAAATTTCGTGCCAGC	CATAGTGGGGTATCTAATCCCAGTTTG	171	Ushio et al. 2017
Mimammal-E	12S	GGACTGGTCAATTTCGTGCCAGC	CATAGTGAGGTATCTAATCTCAGTTTG	171	Ushio et al. 2017
Mimammal-B	12S	GGGTTGGTTAATTTCGTGCCAGC	CATAGTGGGGTATCTAATCCCAGTTTG	171	Ushio et al. 2017
Teleo	12S (L1848, H1913)	ACACCGCCCGTCACTCT	CTTCCGGTACACTTACCATG	63	Valentini et al. 2016
Leray	CO1 (mlCOIintF, jgHCO2198)	GGWACWGGWTGAACWGTWTAYCCYCC	TAIACYTCIGGRTGICCRAARAAYCA	313	Leray et al. 2013, Geller et al. 2013
Vert-16S	16S	AGACGAGAAGACCCYdTGGAGCTT	GATCCAACATCGAGGTCGTAA	250	Vences et al. 2016

The Mifish and Mimammal primers were utilised in one reaction, finally resulting in four separate reactions. Each reaction was done in eight replicates to increase the target DNA used in the reactions. DNA was diluted 5 times to counteract any pcr inhibition from the samples.

SPATIAL COVERAGE AND ENVIRONMENT(S) OF RELEVANCE

eDNA sampling campaigns were performed across 21 UNESCO World Heritage marine sites. All samples were treated the same for DNA extraction and PCR.

PERSONNEL REQUIRED

Highly trained and skilled molecular laboratory scientists/technicians are required to conduct this work.

SAFETY

Working with DNA and in the molecular laboratory requires a high level of awareness regarding safety and the risks associated with chemicals. Additionally, it is crucial to work in a clean environment and to avoid contamination of samples at all times during sample processing. Refer to the guidelines for clean eDNA work.

TRAINING REQUIREMENTS

A specialized training (usually university level) for the molecular laboratory is required for this work.

TIME NEEDED TO EXECUTE THE PROCEDURE

The time needed is highly dependent on the number of samples to be processed. For up to 96 samples, you should reserve about a week for this work.

EQUIPMENT

DURABLE EQUIPMENT

- Pipettes

- Plate centrifuge
- Thermocycler
- Vortex
- Magnetic stand
- Tecan plate reader

CONSUMABLE EQUIPMENT

- Pipette tips
- Ice
- DNA quantification kit (Quant-iT PicoGreen dsDNA kit, Invitrogen, Life Technologies)
- PCR plate
- Aluminum foil

CHEMICALS

Chemicals	Product Name	Quantity	Concentration
Enzyme for amplification	Qiagen Taq mix (2x)	10 µl	
Primer	Primer mix (see above)	0.4 µl	10 µM
PCR-grade water		4.6 µl	
CleanPCR beads	CleanNA	64 µl	
70% Ethanol		200 µl	
TE-buffer		200 µl	
Enzyme for index PCR	MyTaq HS mix (2x)	10 µl	

PREPARATION

All work is to be performed in a clean room specifically prepared for the processing of eDNA samples, and to avoid all possible sources of contamination.

PCR

Step 1: Master mix preparation

- For each sample you need:
  - 10 µl Qiagen Taq mix (2x)
  - 4.6 µl H2O
  - 0.4 µl primer mix (10 µM of each primer)
- Thaw reagents and put on ice
- Prepare a master mix with these 3 components (number of samples + 10%)
- Vortex and spin down

Of the following primers, all Mifish and Mimammal forward primers and all Mifish and Mimammal reverse primers were mixed together before proceeding.

PCR Primer Name	Direction	Sequence (5' -> 3')
Mifish-U-F	forward	GTCGGTAAAACTCGTGCCAGC
Mifish-U-R	reverse	CATAGTGGGGTATCTAATCCCAGTTTG
Mifish-E-F	forward	GTTGGTAAATCTCGTGCCAGC
Mifish-E-R	reverse	CATAGTGGGGTATCTAATCCTAGTTTG
Mimammal-U-F	forward	GGGTTGGTAAATTTCGTGCCAGC
Mimammal-U-R	reverse	CATAGTGGGGTATCTAATCCCAGTTTG
Mimammal-E-F	forward	GGACTGGTCAATTTCGTGCCAGC
Mimammal-E-R	reverse	CATAGTGAGGTATCTAATCTCAGTTTG
PCR Primer Name	Direction	Sequence (5' -> 3')

Mimammal-B-F	forward	GGGTTGGTTAATTTCGTGCCAGC
Mimammal-B-R	reverse	CATAGTGGGGTATCTAATCCCAGTTTG
teleo_F L1848	forward	ACACCGCCCGTCACTCT
teleo_R H1913	reverse	CTTCCGGTACACTTACCATG
mlCOIintF	forward	GGWACWGGWTGAACWGTWTAYCCYCC
jgHCO2198	reverse	TAIACYTCIGGRTGICCRAARAAYCA
Vert-16S-eDNA-F1	forward	AGACGAGAAGACCCYdTGGAGCTT
Vert-16S-eDNA-R1	reverse	GATCCAACATCGAGGTCGTAA

Step 2: PCR plate preparation

- Aliquot 15 µl master mix into each well of the PCR plate
- Add 5 µl diluted purified gDNA to each well (include blank)
- Centrifuge shortly in plate centrifuge (max 1500 rpm).

Step 3: PCR reaction on PCR cycler

Note that there are four different reactions for the four different primer types. Each primer has a specific temperature that is used for the annealing as shown in the table below. Each reaction is performed in eight replicates.

PCR step	Temperature	Duration	Repetition
Initial denaturation	95°C	15 min	
Denaturation	95°C	30 s	35 times
Annealing	Mifish/Mimammal - 61°C Teleo - 55°C CO1 - 57 °C 16S-vert - 65°C	90 s	35 times
Elongation	72°C	60 s	35 times
Final elongation	72°C	10 min	
Cooling	10°C		

Store PCR samples at 4°C for short time storage (1 day) or at -20°C for long time storage. Optional: check products on gel (limited number of samples).

All 8 replicates were pooled and half of the volume was used for magnetic bead purification with the CleanPCR beads (CleanNA).

QUALITY CONTROL, PCR CLEAN-UP

Half of the pooled PCR reaction volume was used for magnetic bead purification with the CleanPCR beads (CleanNA). A total volume of 80 µl from each single locus PCR was used for magnetic bead purification. Final elution was done with 25 µl PCR grade water and stored at -20°C till quantification.

Step 1: Mixing the magnetic beads with the DNA samples

- Homogenize magnetic beads by vortexing.
- Add 0.8x PCR volume (64 µl beads to 80 µl PCR reaction).
- Pipet up and down 10 times to ensure proper mixing.
- Incubate at RT for 5 min.
- Place PCR plate on magnetic stand for 5 min.
- Remove the clear solution while the plate is still on the magnetic stand (5 µl less than the total volume). Avoid taking out any beads.

Step 2: Washing of the beads (plate stays on magnetic stand)

- Add 200 µl of 70% ethanol.
- Incubate for 1 min (longer is no problem, DNA is safe).
- Set pipet at 230 µl and remove everything (discard in special waste container/bottle) o Add 200 µl of 70% ethanol.
- Incubate for 1 min.
- Set pipet at 230 µl and remove everything (discard in special waste container/bottle).
- Remove last traces of ethanol with 10 µl pipet to doublecheck whether all wells are empty (residual ethanol may interfere with downstream analysis).

Step 3: Elution of the purified DNA samples

- Remove plate from the magnetic stand.
- Add 25 µl PCR-grade water.

- Pipet up and down 10 times to ensure proper mixing (avoid air bubbles at the bottom).
- Incubate at RT for 5 min.
- Place PCR plate on magnetic stand for 5 min.
- Prepare new PCR plate (Note: SAME ORIENTATION AS PREVIOUS ONE!).
- Transfer 22 µL to new plate (to be sure no beads are transferred, although they do not form a problem later on).

QUALITY CONTROL

Concentration was measured with the Quant-iT PicoGreen dsDNA kit (Invitrogen, Life Technologies) according to the manufacture's guidelines.

Step 1: Prepare 1 x TE buffer

- 200 µl needed per sample.
- For 100 samples prepare 20 ml 1xTE buffer:
  - 1 ml 20xTE buffer
  - 19 ml high pure water
- Store in falcon tube at RT (long-term storage).

Step 2: Prepare standard dilution series

- Label 6 eppendorf tubes.
- Pipet 1xTE buffer first, no need to change filter tip.
- Mix by pipetting:
  - Tube 1 = dilution (1/100; 1 ng/µl) = 198 µl 1x TE + 2 µl of Lambda stock DNA (100 ng/µl)
  - Tube 2 = dilution (1/2; 0.5 ng/µl) = 100 µl 1xTE + 100 µl tube 1
  - Tube 3 = dilution (1/2; 0.25 ng/µl) = 100 µl 1xTE + 100 µl tube 2
  - Tube 4 = dilution (1/5; 0.05 ng/µl) = 160 µl 1xTE + 40 µl tube 3
  - Tube 5 = dilution (1/5; 0.01 ng/µl) = 160 µl 1xTE + 40 µl tube 4
  - Tube 6 = Blank (1) = 100 µl 1xTE
- Pipet 100 µl of each tube into Greiner plate.

Step 3: Prepare samples

- Pipet 99 µl 1xTE buffer (prepared in step 1) into Greiner plate.
- Add 1 µl DNA sample (= 1/100 dilution).

Step 4: Prepare Quant-iT reagent

- Note: Prepare reagent just before use!
- 100µl needed per sample and standard.
- For 100 samples prepare 10 ml Quant-iT reagent:
    - 50 µl 200x Quant-iT reagent (blue cap)
    - 9950 µl 1xTE buffer (prepared in step 1)

Step 5: Add Quant-iT reagent to samples and standard dilution series

- Add 100 µl Quant-iT reagent to each well of the Greiner plate (samples and standards) with multipipet.
- Wrap plate in aluminium foil and measure within 5 – 10 minutes.
- Measure with Tecan plate reader.
- Calculate concentrations.

POOLING OF THE DIFFERENT LOCI PER SAMPLE AND INDEX PCR

As four different reactions were performed for each sample, these reactions are pooled prior to indexing. Unique indices are then added for each sample.

Step 1. Pooling and aliquoting of index primers

- Five µl from each purified locus PCR sample was pooled before index PCR.
- Combination of 96 Unique dual index primers for each plate.

2. Step 2_Master mix preparation

- For each sample you need:
    - 10 µl MyTaq HS mix (2x)
    - 8 µl water
- Thaw reagents and put on ice.
- Prepare a master mix with these 2 components (number of samples + 10%).
- Vortex and spin down.

Step 3: PCR plate preparation

- Aliquot 18 µl master mix into each well of the PCR plate.
- Add 1 µl of the unique dual index primers to each well (2.5µM each) with multi-channel pipet.
- Add 1 µl pooled purified PCR's to each well with multi-channel pipet
- Centrifuge shortly in plate centrifuge (max 1500 rpm).

Step 4: PCR reaction on PCR cycler

PCR step	Temperature	Duration	Repetition
Initial denaturation	95°C	1 min	
Denaturation	95°C	15 s	10 times
Annealing	51°C	15 s	10 times
Elongation	72°C	10 s	10 times
Final elongation	72°C	5 min	
Cooling	10°C		

Remove plate from PCR cycler when reaction is finished!

Each PCR reaction is followed by a magnetic bead purification (0.8 x ratio). Final elution is done with 25 µl pure water and quantified with the picogreen kit as before.

Step 5: Pool all samples for sequencing library

Pool 20 ng of each sample. Add full volume of low concentrated samples.

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