LEIBNIZ CENTRE
for Tropical Marine Research

ZMT

KENYA MARINE AND FISHERIES RESEARCH INSTITUTE
K.M.F.R.I.

# Lab protocols for Fish Barcoding

*Achim Meyer, Anissa Volanandiana, Cretus Joseph Mtonga, Debora Benjamen, Diana Karan, Erica Tovela, Humphrey Mahudi, Janine Baxter, John Kochey Kipyegon, Kennedy Oduor, Levy Michael Otwoma, Liban Isse Farah, Manda Kambikambi, Mattia Ghilardi, Mpilonhle Nyawo, Nathan Mrombo, Oscar Puebla, Said Mgeleka, Samuel K.K. Amponsah, Segun Olayinka Oladipo, Sonia Bejarano, Teresia Wacira, Tsiaranto Fanoro and Valarie Silali*

Protocols Series No. 1 May, 2023
https://doi.org/10.21244/zmt.2023.002


# Preface

The ongoing biodiversity loss due to anthropogenic activities is alarming. While some effects are driven by climate change, local factors and direct exploitation in particular of fish can contribute considerably to the observed effects. Therefore, ecosystem management can benefit from regional action which should be based on data-driven decisions. For example, environmental DNA (eDNA) analysis allows now efficient biodiversity screening of coastal ecosystems, but relies on regional reference databases which are sparse for many tropical countries. Also in functional ecology sequence information can help to better understand environmental changes. This manual is a contribution to promote molecular reference library compilations in the Western Indian Ocean and foster the application of molecular species identification in general. Due to their great economic and ecological importance we focus on fish.

In 2022, the ZMT Academy initiated a third party funded (Volkswagen Foundation) capacity building activity on biodiversity research at the East African coast. The resulting workshop Fish Barcoding and Functional Ecology: Monitoring the Status of Marine Coastal Ecosystems was jointly organized by the Leibniz Centre for Tropical Marine Research (ZMT) and the Kenya Marine and Fisheries Research Institute (KMFRI). The goal of the workshop was to deliver state of the art laboratory practice combined with networking activities strengthening inner African cooperation, international collaboration and individual scientific careers. This manual is a direct output from the workshop.

# Content

# Introduction

This manual focuses on applied molecular methods in reef fish barcoding. The functional ecology component of the workshop, which focused on the extraction of morphometric traits from fish images using MorFishJ v0.2.1 (Ghilardi 2022), a plugin for ImageJ, and subsequent statistical analysis in R are not included in this manual, because these steps are well documented elsewhere. A second manual describing the analysis of barcoding data is planned. Here we focus on the laboratory work of fish barcoding for submission of reference pictures and sequences to databases, in particular the Barcode Of Life Database (Ratnasingham and Hebert 2007). The mandatory paperwork in preparation for barcoding activities may be demanding and is context-dependent. Required documents can include an ethical statement/animal welfare approval, research permit, PIC (Prior Informed Consent) and MAT (Mutually Agreed Terms) -both documents are related with the Nagoya protocol on access and benefit sharing-, confirmed completion of the lab safety instruction, and possibly others such as CITES (Convention of International Trade on Endangered species of wild fauna and Flora) restrictions, custom regulations or veterinarian obligations.
A comprehensive safety instruction is likewise beyond the scope of this manual, but we want to remind for good lab work practice. A safe working environment should be created before starting the practical work. At ZMT, 1 % Incidin™ is the standard cleaning detergent (only spray on and wipe off with a paper towel) for "coarse" dirt. Use hypochlorite to obtain "DNA-free" surfaces (spray 5% bleach, 10min exposure time, then wipe away with clean water and paper towels). Surfaces for microbiological work are cleaned with Bacillol® at ZMT, but 100% 2-propanol or 70% ethanol could also be used (spray on and wipe off with paper towel). Floors can be cleaned with 10% Microbac®.

All chemical solutions made and stored in the laboratory must be labelled with date, content (including risk and safety pictograms) and your initials. At your lab supplier or here (https://ehs.utoronto.ca/our-services/chemical-and-lab-safety/whmis/whmis-workplace-labeling/workplace-label-templates-for-commonly-used-chemicals/) you find printable workplace label templates with the required risk and safety pictograms for commonly used chemicals. Within this manual risk and safety advice is printed in magenta. # Comments are given after a hashtag (#).

# Fish specimen documentation

For reliable molecular reference data, high quality documentation of the whole fish is mandatory. This chapter focus on the photo documentation, but some other data should be collected during sampling. For example, according to Steinke and Hanner (2011) the fish barcode of life (FISH-BOL) entries recommends the subjects below to be recorded:

- Collectors name
- Collection date (dd-mmm-yy) and time
- Locality: latitude and longitude using the World Geodetic System 1984, and coordinates are in degree decimal-degree format (e.g. 72.098–114.84), and the FAO region (recorded as a structured comment in the Extra Info field at BOLD).
- Depth in meters.
- If possible, collection gear (sampling method/effort), vessel
- Notes on habitat, microhabitat, and associations
- Sex of specimen
- Life stage (adult, juvenile)
- Identifier such as unique sample code
- Fixative

As soon as available add comprehensive information on the institution where specimens are vouchered and accession/catalog number (Museum voucher ID). Type status for new species (holotype/paratype). Preservation method (ethanol, DESS, …), tissue type, …

### General notes of caution when handling fish:

Be aware that some dorsal fin spines might be very sharp and in some fish such as stonefishes and lionfishes contain venom (also very small juveniles). Surgeon fishes and rays have razor sharp spines on the tail and opercular spines can be sharp in squirrelfishes and angelfishes, or even venomous as in toadfishes. In case of an accident, calm the injured person if necessary, clean and disinfect the wound. If necessary, consult a doctor after providing first aid and use abrasives, sedatives or antihistamines. There are reports that a hot water bath can reduce pain. Therefore the water bath is adjusted to the highest temperature that the skin is able to tolerate, check the temperature with the elbow before dipping the hand to avoid burnings (Darlene and Phee-Kheng 2013).

## Documentation of fish specimens at the fish market

Together with the documentation, tissue samples are taken and preserved for molecular work. Therefore, it is mandatory to read the manual until page 14 (DNA Extraction) before you plan your sampling.

### Things to do before moving to the fish market

a. Arrange with the fishers or fisheries technical officers to know the time of landing before the day of sampling.
b. Know the types of species you are going to sample at the fish market as these may vary with the gear type, type of fishery and seasons. For very rare species you may ask several people at the market to notify you whenever they see it.
c. Be at the fish market early to get fresh material and avoid missing fish samples.
d. If possible, go with at least one native person who understands the language spoken by the fisherman/woman. Ideally, the person will assist with communication, the imaging of the fish samples and metadata collection.
e. Read carefully **all subsequent steps** including tissue fixation (until page 14!) **in advance of your sampling**.

### Material for fish documentation at a fish market

- First aid kit
- High quality camera (or smartphone)
- Ruler or other length standard tag
- Notebook or lab book.
- A lead pencil and paper
- Permanent markers
- Optional, mostly depending on the employed fixative: Lab coat, goggles and gloves.
- Optional: Audio recorder to reduce the need of writing while sampling.
- Optional: Color reference e.g.: Calibrite ColorChecker Classic (Mini or Nano).
- Optional: Measuring plate with standard size tag (and color card) where you place fish and may position the dorsal fin with needles.


Figure 1: Fish market sampling. Panel A: Labelling of tubes on site (Humphrey Mahudi on the left) and taking pictures vertical from above (see Mpilonhle Nyawo in the middle). Panel B: The ruler is placed horizontally below the fish and the dorsal fin might be positioned manually. See notes of caution for spines page 5. Panel C: Label with a lead pencil on paper to include an additional label within the tube of the fin clip to supplement the outside labelling. Note the recommended O-ring in the lid to minimize evaporation.

## Protocol for fish documentation at a fish market

1) Ask for consent to photograph fishes and taking fin clips. Take your time to explain your goals and methods carefully. Wait for consent before you start.
2) When more fishes than needed are available select the freshest specimen (Gills should be dark red with translucent mucus. Over time gills become bright red or pink and later brown, mucus has a succession to opaque and later clotted). Keep on ice when possible.
3) Place the fish in a straight orientation.
4) Position a ruler horizontally below the fish as a reference for measurements on pictures. You may include a color reference for standardization as well.
5) Use a white board/cardboard to spread out the fins using pins. Alternatively spread out the dorsal fin with your hands (*See notes of caution page 5*).


Figure 2: Straight orientation with ruler and carefully erected dorsal fin.

6) Write the ID and, if known, the species name on a piece of paper and place next to the fish.
7) Take a high resolution picture horizontally and centered above the fish (figure 2). Check the image quality carefully by zooming in. Repeat if you see shortcomings.
8) Make an empty picture after the last photo from the same specimen to facilitate screening of pictures later on.
9) Note the order of good images taken using the image ID and additional information, such as local/common English name, where the fish was caught, which gear was used, the time the fish was caught and others in your notebook or lab book. In the absence of a conducive situation, recordings can be done using an audio recorder such as a smartphone and be transferred to your notebook after the sampling.
10) Proceed with tissue sampling for DNA analyses.

Table 1: Example table for catch data from the Mombasa old town fish market with common names in both Kiswahili and English. Your data might be modified or extended (e.g.: sales prizes), depending the specific research goal.

| NO | LOCAL NAME | ENGLISH NAME | SCIENTIFIC NAME | FISHING METHOD | CATCHMENT AREA | IMAGE(S) | ..... |
|---|---|---|---|---|---|---|---|
| 1 | Kiboma | Yellow fin tuna | *Thunnus albacares* | Line | Pemba Canel | DSC_4849.jpg, DSC_4852.jpg | |
| 2 | Fute (grey) | Sweetlip | *Plectorhinchus flavomaculatus* | Trap net | Nyali | 1edf1613-9a61-5ea05c20c2c0.jpg | |
| 3 | Tafi | Shoemaker spinefoot | *Siganus sutor* | Beach Seine | Bamburi beach | DSC_4849.raw | |
| 4 | Tatau | Sea bream | ..... | ..... | ..... | .... | |

## Documentation of fish specimens in the laboratory

Depending on the goal, for fish up to 30cm an office scanner such as Epson EP44859 can be used following the CCBD Methods Release No. 3 protocol according to Steinke et al. (2009). For high resolution pictures of cryptobenthics or other fish below 15cm a camera system is preferred.

### Material for fish documentation in the lab

- a digital camera system with macro lens and sufficient light such as unleashed flashlights;
- non-fluffing paper towels (KimWipes);
- a balance with an accuracy of 0.001 grams to weight each fish;
- a photo aquarium with non-attached white or black plastic sheet (eg acrylic box, see figure 3B). Acrylic sheets to be glued with Glue Acrifix 192. # hardens with UV light:

  All panes 3mm thickness
  2x 16cm x 12cm (front pane and back)
  1x 16cm x 7,4cm (bottom plate)
  2x 11,7cm x 7,4cm (sides)

  Black and white PVC background plates
  15,4cm x ~17cm

- a ruler or size tag on the plastic sheet

- a paintbrush or wooden stick
- a lead pencil and paper
- permanent markers

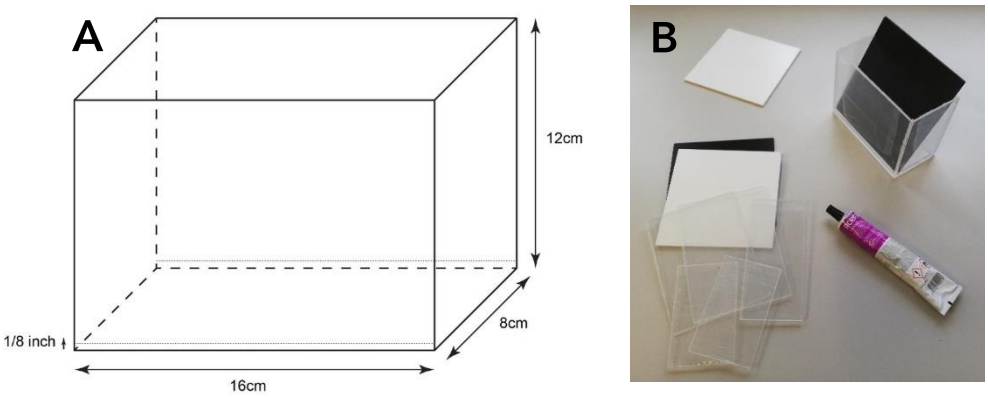


Figure 3 panel A: Dimensions of the photo aquarium (acrylic box 16cm x 8cm x 12cm (L x D x H) or glass aquarium. Panel B: Acrylic sheets, PVC sheets and the glue needed to build a small photo aquarium on site. Do not glue the black and white PVC sheets.

## Protocol for fish documentation in the lab

Receive living or freshly killed fish (same day) in chilled seawater or larger specimen on ice to preserve coloration as good as possible.

A. Use an office scanner following Steinke, Hanner et al. (2009)
B. Use an aquarium, camera and flash light assembly as shown in Figure 4.

1) Place the fish in the photo-aquarium with seawater.
2) Correct patiently the position using a paintbrush or wood stick until the fish is horizontal and fins are spread.


Figure 4: Imaging in the lab. Panel A: Said Mgeleka positioning a cryptobenthic reef fish for documentation. Panel B: Side view of the photo set up. In the background the next fish is prepared.

3) Take one or several pictures and note the name(s) of your image(s).
4) Take another picture with the identifier before proceeding to the next specimen (or an empty picture).
5) Remove the fish from the photo-aquarium and "dry" using KimWipes.
6) Weigh the fish and note the value in your data sheet.

# Tissue sampling

Safety: As for all laboratory work pay attention to self-protection, thus if handling chemicals you wear gloves (and lab coat). However, if sampling on a fish market wearing gloves and lab coat may scare the vendor and/or customers and is not mandatory if working on healthy fish and using nonhazardous chemicals such as ethanol. If you feel reservations by the fish market community, you need to consider to skip the gloves to get the consent for working and to be able working cooperatively. If using DMSO, gloves are mandatory but the fixative can be added after the tissue has been sampled. Cover the tissue with DESS within the tube when outside the fish market and away of any food processing area.

## Material for tissue sampling

- 2ml cryovials with O-ring for the samples
- Tweezer
- Scissor or scalpel
- Fixative (such as abs. ethanol, DESS, or a commercial solution such as DNA shield (Zymo Research R1100-50)
- Paper and lead pencil
- Permanent marker
- Cellotape
- Fixative solution e. g. absolute ethanol in a spout wash bottle
- Soap water (or 5% bleach)
- Paper towels

## Protocol for tissue sampling

1) Label prefilled cryovials with a unique identifier on the outside using a permanent marker. If working with students you may prefill 1.5 mL snap-cap tubes with 1.3 uL of 96% ethanol and explain that if the tube overflows, they have collected too much tissue.
2) Protect the labelling with sellotape (in particular important for the ethanol tubes which must have an additional pencil paper label, see below point 4.).
3) Document the specimen as described above (pages 2-6).
   Use tweezers to handle the fin and cut fin clips with a scissor or scalpel (see Figure: 5A). The **fixative/tissue ratio must not exceed 10:1**. Cut up to 100mg tissue, but usually take much less such as:
   a) Fin clips 4mm x 4mm up to 1cm$^2$
   b) One or more gill arches with attached filaments (see Figure 5B)
   c) The right eye for very little juvenile fishes below 15mm
   d) Small cubes from the lateral muscle (use the right side of the specimen)
   e) The whole fish for larvae (ideally series of conspecifics are available for fixation in formalin for standard morphological analysis)

4) Transfer the tissue to a 2ml cryo vial with O-ring using a tweezer. (If using tubes without sealing, parafilm helps provisionally to reduce evaporation - not recommended).

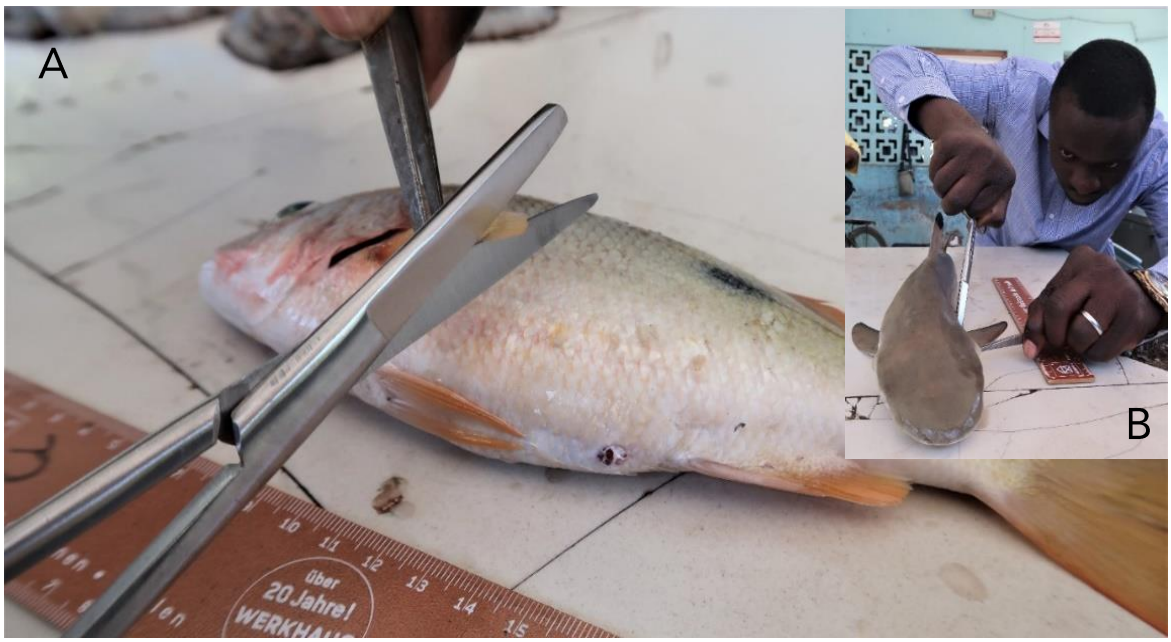


Figure 5: Taking tissue samples for barcoding. Panel A: Cutting up to 1cm fin clip. Panel B: Segun Oladipo taking a gill sample.

5) Clean the equipment used after each sample collection using soap water and disposable paper towels as this will minimize cross-contamination of fin-clip samples. 70% ethanol can be used for disinfection, but has no effect on DNA take over. 5% (-10%) bleach (sodium hypochlorite) might be used to break down contaminating DNA molecules, but be careful to not destroy your next target DNA as bleach is very reactive. Wipe all remains carefully with distilled water after bleach exposure.
6) Label paper with a lead pencil and place the label in the tube.
7) Close the tube and place in a storage box. Label the lid for faster screening.

# Tissue preservation

Optimal tissue preservation is the prerequisite for high quality DNA extraction and thus the precondition for reliable results in PCR and sequencing. Tissue type is less important than the post mortem interval, thus try to sample quickly. In fish DNA integrity is good within the first 24h after death (depending on environmental conditions as well) but very poor after four days (Rodríguez-Ezpeleta et al. 2013). See also above for estimation of time of death (Protocol for fish documentation on a fish market, step 2). A rule of thumb to keep DNA intact is:

*Dry, dark and cold is* ***good*** *– warm, water and (UV!)-light is* ***bad****.*

Keep samples in the dark, particularly protected from direct sunlight and possibly in a cool place. There are different fixation methods available (Nagy 2010), but here we compare only the two fixatives used predominantly with fish tissue a) ethanol and b) DESS (**D**MSO, **E**DTA,

**S**alt **S**olution). However, a good alternative is to use commercial solutions such as DNA/RNA shield (Zymo Research R1100-50) if available.

Table 2: Comparison of advantages and disadvantages of ethanol and DESS buffer for fin clip (or other tissue) storage. The beauty of ethanol is the ease of use whereas DESS has the benefit of much higher final DNA concentrations in cost effective extraction methods (+ high - low).

| Properties | Ethanol | DESS |
|---|---|---|
| DNA quality and quantity | ++ | +++ |
| Availability of fixative | +++ | + |
| Handling | + | -- |
| Widespread use | +++ | - |
| Cost | +++ | ++ |
| Transport | - | ++ |
| Evaporation | ++ | - |
| Flammable | +++ | - |
| Easy to combine with the cost effective salting out protocol | - | +++ |
| Documented DNA preservation for long term storage | ++ | - |
| Long term storage maintenance needs | ++ | - |
| Health hazard | - | + |
| Tissue morphology unaffected | + | - |

## Ethanol

Ethanol is the most commonly used fixative and readily available. The main disadvantages are the high evaporation rate, flammability and the unfavorable interference of alcohol with some extraction methods such as the cost effective salting out protocol. Ethanol drives water out of the cells leading to coagulation of proteins as the main preservative effect; thus ethanol prevents enzymes such as DNAse to work.

In ethanol fixed samples DNA hydrolysis and enzymatic activity is prevented by desiccation, thus the replacement of tissue water by diffusion and dilution with ethanol, highlighting the importance of changing ethanol 24h after the initial preservation. 94–98.9% EtOH is recommended as a fixative, but traces of denaturing agents (e.g. isopropanol, acetone, methyl ethyl ketone, and gasoline) usually do not significantly alter the performance (Nagy 2010). With 70% ethanol, a high degree of degradation has been observed. 30% water is easily reached if the tissue is over the recommended 1:10 (tissue/ethanol) ratio or ethanol replacement after the initial fixation was postponed or not carried out. The optimal concentration for DNA preservation is about 95–99% in your storage tube. Ethanol is prone to evaporation thus sampling tubes must be sealed and regular maintenance to replace evaporated ethanol is mandatory for long term storage.

Be aware that fridges need to be explosion-proof to allow storing quantities (>100ml in total) of ethanol. To facilitate air transport, ethanol might be discarded in advance of the shipment and immediately replaced after arrival (transport of ethanol soaked tissue), or ethanol is reduced below 70% during transport to apply another packaging group (Packing Group III) with fewer restrictions and replaced to full strength immediately after arrival.

Air Transport

If air shipping ethanol IATA (International Air Transport Association) Special Provision SP A180 applies.

- Specimens are placed in vials or other rigid containers with no more than 30 mL of alcohol or an alcohol solution;
- The prepared specimens are then placed in a plastic bag that is then heat–sealed;
- The bagged specimens are then placed inside a another plastic bag with absorbent material then heat sealed;
- The finished bag is then placed in a strong outer packaging with suitable cushioning material;
- The total quantity of flammable liquid per outer packaging must not exceed 1 L; and
- The completed package is marked "scientific research specimens, not restricted Special Provision A180 applies".
- The airline may have additional restrictions.

Box 1: IATA Special provision SP A180 for the transport of non-infectious specimens such as fish fin clips in alcohol ≥70% (UN1170, Class 3, packing group II).

### Material tissue preservation ethanol

- See 1. - 6. from the general sampling part above (p.9).
- Ethanol 96% - 98.9% (e.g.: Roth #9065.3)
- Freezer or deep freezer for long term storage such as years or decades but avoid household devices for safety reasons.

### Protocol for tissue preservation in ethanol

1) Follow 1. - 6. from the general sampling part above (page 8).
2) Label the tube and protect the label with sellotape.
3) Fill the cryovial with ethanol using a spout wash bottle.
4) Use tweezers to handle the fin, cut a small piece of tissue from the fin using scissors. Cut up to 100mg tissue. The ethanol/tissue ratio must not exceed 5:1.
5) Write your unique sample identifier with the lead pencil on paper and put it into the vial.
6) Put a short code on the lid for rapid screening of the box.
7) **Change the ethanol on the next day**! # Fin clips are no watery tissue; thus 1 change is sufficient. Whole fish will need a second exchange after one or two weeks because ethanol is constantly diluted by water released from the tissue as explained above.
8) Store cool or cold, if possible at -20°C to -80°C for long term storage (decades and more). Use explosion-proof fridge or freezer only.

## DESS (20% DMSO, 0.25 M EDTA, sodium chloride saturated solution)

DESS has been shown to preserve vertebrate DNA more effectively than ethanol (see e.g.: Robertson et al. 2013). The DESS disadvantages are the need for advance preparation, including difficulty to dissolve EDTA (see recipe below). DMSO (Dimethylsulfoxid) is not readily available and has modest health risks as DMSO perforates cell walls, thus can introduce other unwanted substances into human cells: If skin contact with DESS occurs, the affected area should be washed thoroughly with clear running water. At room temperature, DMSO is noncorrosive and nonexplosive. Therefore, no special regulations apply to the transport of DESS by air according to IATA (International Air Transport Association). However, some airlines have general restrictions for the transport of liquids (Kilpatrick 2002). DMSO helps to penetrate

cells, thus to get EDTA (ethylenediaminetetraacetic acid) in contact with the DNA within the cell. The actual DNA preservation is done exclusively by EDTA protecting DNA from degradation (Sharpe et al. 2020).
NaCl binds excessive water and neutralizes the DNA charge (DNA's sugar phosphate backbone is charged) to make the molecule less reactive. The salt also helps remove proteins bound to the DNA and keep these proteins dissolved in the water.

### Material tissue preservation DESS

- See 1. - 6. from the general sampling part above.
- 20% DMSO (dimethyl sulfoxide)
- 0.25M EDTA (Ethylenediaminetetraacetic)
  a) *Recommended*: Disodium EDTA MW 372.24. Adjust to pH 8.0 with NaOH pellets (ca. 20g/L). # $Na_2EDTA$ is MUCH easier to work with than anhydrous EDTA.
  b) Anhydrous EDTA MW 292.24. Adjust to pH 8.0 with NaOH pellets (ca. 42g/L) *Not recommended as more than double amount of base is needed to shift pH to 8 and complete dissolution of EDTA takes much longer than Na-EDTA.*

  # With both EDTA versions pH will be around 3 when EDTA is introduced into distilled water. Instead of using NaOH pellets it is faster to work with liquid 0.5M NaOH as EDTA will not completely dissolve until the pH reaches 8. Additionally, you may want to speed up EDTA entering into solution by heating to 30°C or up to 50°C; but in any case you need to be patient getting EDTA dissolved completely.
- Deionized water
- Enough NaCl to saturate the solution. You will need about 150-200g/L

Optional: Prepare a 2.5M NaOH solution to pH a 0.5M EDTA solution to 8.0:

1) Weigh in 30 g NaOH (MW 39.997) and add to a 500 mL beaker.
2) Add 250 mL of deionized water to the beaker while stirring. Caution, the solution becomes hot.
3) When cooled, transfer to a 500 mL graduated cylinder. Fill up to 300 mL using deionized water.
4) Transfer to a 500 mL sterile Duran bottle. Label with batch, date and your initials.
5) Store at room temperature.

Prepare the 0.5M EDTA solution

1) Weigh 93g $Na_2EDTA*2H_2O$ in a 500ml beaker. # Make sure to use disodium EDTA salt (MW 374.24) otherwise much more NaOH is needed to lower pH.
2) Add 300ml deionized water.
3) Add a stir bar and stir.
4) Bring pH to 8.0 using the 2.5 NaOH solution (preferred) or NaOH pellets.
5) EDTA will begin to dissolve at a pH above 7, be very patient. Heating up to 50°C may help to speed up the process.
6) When completely dissolved fill up to 500ml with deionized water.
7) Transfer to a 500 mL sterile Duran bottle. Label with batch, date and your initials.

8) Store at room temperature.

### Protocol for tissue preservation in DESS

1) Prepare 250ml DESS solution:

    1. Add 125ml 0.5M EDTA in a 500ml beaker.
    2. Add a stir bar and stir.
    3. Add 50ml Dimethyl Sulfoxide (DMSO)
    4. Add Sodiumchlorite (NaCl) until it does not dissolve any more (40-50g, be patient).
    5. Fill up to 250ml with deionized water.
    6. Transfer to a sterile Duran bottle or into collection vials. Label properly.
       # Precipitation might be observed, but this is wanted in a supersaturated solution.

2) Follow 1. - 6. from the general part for tissue sampling on page 9. Do not forget to label the tube with a unique identifier on the outside.
3) Fill the cryovial with 1.5ml DESS using a pipette or spout wash bottle. (Fume hood, goggles, gloves, lab coat).
4) Use tweezers to handle the fin and cut a small piece of tissue from the fin using scissors. Cut up to 100mg of tissue.
5) Write your unique sample identifier with the lead pencil on paper and put it into the vial.
6) Put a short code on the lid for rapid screening of the box.

No change of DESS is needed on the next day.

# DNA extraction

## Introduction

Establish a pre-PCR area for DNA extraction. Assign different working stations (benches) within the Pre-PCR lab for different tasks to separate DNA extraction and PCR set-up. Assign equipment to one area and do not move. In particular, Post-PCR items cannot enter the Pre-PCR area again. In sensitive applications (low amount of DNA, population genetics), be particular careful with cross contamination via your lab-notebook and pen as well. If the suggested spatial separation is not possible, at least different sets of pipettes should be designated for different procedures and filter tips used at the pre PCR. You may bleach your workspace to remove traces of foreign DNA (Nilsson et al. 2022) before you start, but make sure you have good ventilation and wear protective equipment (goggles, gloves & coat). 1% sodium hypochlorite and 70% ethanol form dangerous gaseous chlorine and chloroform when combined; or even worse the most widely used extraction kits contain guanidine thiocyanate or guanidine hydrochloride, which are reactive with sodium hypochlorite to produce chloramines and hydrogen cyanide gases. Thus, carefully remove bleach after an exposure time of about 10min with bidest water and paper towels as you may destroy your samples when bleach is carried over to your equipment.


Figure 6: Spatial Separation of working areas in different rooms at ZMT (photos Tom Vierus). Left: Pre PCR lab with UV hood to set up PCR reactions. No genomic DNA is allowed within the hood, but the extraction area is in the same lab but on another bench. Right: Post PCR lab with gel documentation. No transfer of any equipment from Post-PCR to Pre-PCR. Be careful with your lab book and lab coat as well.

Here we describe two DNA extraction protocols. A) the Chelex protocol because it is easy to apply and B) the Salting Out protocol which combines a very cost effective extraction method with the efficient DESS fixation. In case you have difficulties due to low tissue quality, please switch to a glass-fiber based system such as commercial kits or consider the economical 96well glass fibre method by Ivanova et al. (2006). The two presented protocols use proteinase K from GeneOn which has been tested for possible in proteinase K activity reduction at higher temperatures. There was no significant loss in activity after 24h at 60°C and so far we did not observe issues due to long distance transport without cooling.
For each round of extractions process one tube without tissue is to be used as negative control in subsequent PCRs. After the extraction use 5-10µl of the undiluted extraction on an agarose gel for qualitative and quantitative control of the gDNA. Photometric DNA quantification is also helpful and fast (e.g. µCuvette G1.0 Eppendorf). Use the negative control as blank.

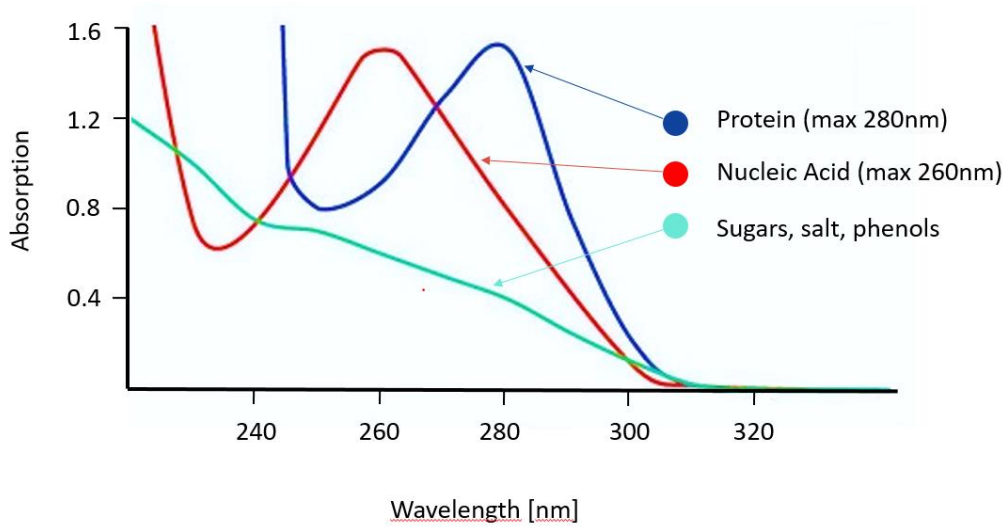


Figure 7: Photometric spectra of main extraction components. Photometer usually calculate purity ratios: A260/A280 ~1.8 indicates pure DNA (below 1.8 may suggests contamination with proteins). Expected A260/A230 values are commonly in the range of 2.0-2.2 (below 2.0 may suggest contaminants such as organic compounds including proteins).

## Chelex extraction

The Chelex extraction is easily adoptable to high throughput extractions. If working with 96 well plates without available plate centrifuge you may build your own from a salad spinner (Morán and Galindo 2011).

### Material Chelex extraction

- Soap water
- non-abrasive towel (KimWipes)
- Chelex 100 Resin (Biorad #1421253)
- Proteinase K, 20mg/ml (GeneON # 405-001) # KEGG Enzyme 3.4.21.14
- Pipettes (20µl, 200µl and 1000µl)
- Vortex mixer
- Small table top centrifuge (or homemade salad spinner plate centrifuge)
- Forceps
- Sample tubes
- Deionized water
- 10ml measuring cylinder
- 50ml or 25ml beaker
- Small stir bar
- Magnetic rod
- Scale

### Protocol to make the Chelex stock solution (10% wt/vol)

1) Weight 1 g of Chelex
2) Measure 9 ml of deionized water into a 25ml beaker containing a small stir bar.
3) Place the Chelex solution on the magnetic stirrer for homogeneous mixing of the solution.

The Chelex solution can be stored for years at ambient temperature e.g. in a parafilm sealed falcon tube.

### Protocol Chelex extraction

1) Pipette 195 µl of the stirred (5% -) 10% Chelex solution into a 200µl extraction tube, stripe or 96 well plate. The Chelex resin beads are heavy and need to be constantly stirred in order to maintain uniform distribution during withdrawal. Therefore, pipet the volume needed for each individual sample (195µl) directly from the beaker while the stir bar is mixing. Due to the coarse Chelex powder the pipette tip used must have a relatively large bore. 1000-µl pipet tips are adequate or use wide-bore pipette tips.
2) Clean forceps with soap (or 10% bleach), rinse with water and dry with a non-abrasive towel.
3) Remove the preserved fin clip from the sample tube using the forceps and rinse with distilled water to get rid of the fixative.
4) While holding the fin clip, cut a piece of it such as 1mm$^2$ tissue and place in the extraction tube with the Chelex solution. You may crush it into small pieces to increase

the surface area for digestion. Label the tube and take notes in your lab book. If using a 96 well plate be focused, as it is easy to mess up.

5) Place unused tissue back in the sample tube for further storage.
6) Pipette 5 µl of the protease K solution to the extraction tube containing both the fin clip and the Chelex solution. However, it is possible to add proteinase K before the tissue as it has been shown that activity does not decrease by self-digestion in higher enzyme concentrations (Bajorath et al. 1988).
7) Close the lid or seal the plate. Note: Aluminium foils are difficult to remove; better use plastic foil for plates instead.
8) Vortex briefly.
9) Shake down droplets from the lid or tube wall, or spin down.
10) Incubate at 60°C for 30min (max 200min) or at 37°C overnight on a thermocycler (tissue digestion).
11) Incubate at 96°C for 10min to deactivate proteinase K.
12) Briefly place the sample tubes on the vortex mixer for uniform mixing.
13) After vortex mixing, centrifuge at 10 000g for 5 min to collect Chelex and any other remaining solids on the bottom of the well.
14) Pipette 100 µL of the supernatant to a sterile tube and store at 4°C until agarose quality control and/or PCR testing. Usually a dilution of 1:10, 1:20 and 1:50 is used as a PCR template. For long term storage of the undiluted DNA stock use a freezer (≤ -20°C).

## Salting Out extraction

### Material Salting Out extraction

- 3M NaCl solution
- Tris-HCl
- 0.5M EDTA (see page 8)
- Proteinase K
- Chilled absolute ethanol (-20°C)
- 70% ethanol
- sterile $H_2O$
- RNAse (optional)
- Microcentrifuge capable of at least 10.000g
- Vortexer
- Heating block, preferably with shaking

Table 3: Reagents of the extraction buffer for different final volumes.

| Reagents | Final volume 50mL. | Final volume 200mL |
|---|---|---|
| EDTA 0.5M | 1 mL | 4 mL |
| 1M TRIS-HCl (pH 7.5) | 5 mL | 20 mL |
| NaCl 3M | 5 mL | 20 mL |
| SDS 20% (w/v) | 5 ml. | 20 mL |
| $H_2O$ | Fill up to 50ml (34 ml) | Fill up to 200ml (136 mL) |

### Protocol Salting Out

1) Prepare the fin clip or other tissue by eliminating all alcohol completely by evaporation on a heating block at 65°C. DESS preserved samples are removed from the sample tube using forceps, rinsed and waved in deionized water to get rid of the fixative.
2) Add 200ul of the extraction Buffer and 5ul of proteinase K (spin down the proteinase before opening as some liquid may stick to the walls or lid);
3) Incubate at (56°-) 60°C for 1-3 hours (or overnight at 37°C) preferably by shaking and vortex occasionally;
4) Spin down after the incubation;
5) Add 450ul of 3M NaCl and vortex;
6) Centrifuge at ≥10000g for 15 min to collect cell debris;
7) Transfer the supernatant (contains DNA) in a sterile 1.5ml tube without touching the pellet; (# it is preferred to get less volume but higher purity, thus be generous to leave liquid in the tube. Up to 500µl might be transferred. If particles were accidentally transferred, repeat centrifugation and decanting);
8) Add cold (-20°C) absolute EtOH to fill the 1.5ml tube (about 650µl);
9) Let DNA precipitate overnight (minimum 30min) in a freezer at -20°C;
10) Centrifuge at ≥10000g for 20 min;
11) Eliminate the EtOH by pipetting. Do not touch or disturb the pellet (pellet contains DNA). Be careful as the pellet is often not visible. Trust it is there and place the hinge of the tube to the outside position to have a mark for guessing the position of the pellet at the bottom side of the tube. If budget allows, you may stain the pellet for better visibility (e.g.: Merck pellet paint # 69049-3 or Roboklon vivid violet # E4502-01);
12) Wash the pellet using 700ul of EtOH 70% and mix inverting the tube;
13) Centrifuge at ≥10000g for 20 min;
14) Gently remove the ethanol by pipetting without disturbing the pellet;
15) Let the pellet dry at 50°C on thermoblock or at RT; (# Do not over dry, as resuspension may become difficult. Usually, 10min of drying is sufficient if all liquid is removed with a pipette. Check for droplets at the wall of the reaction tube and proceed when they are gone);
16) Re-suspend the DNA in 50ul of Deionized water (molecular grade) or TE buffer (10mM Tris, 1mM EDTA, pH 8.0);
17) Keep the DNA at +4°C if used during the same week of extraction, otherwise keep frozen at -20°C.

# PCR: Amplification of the COI gene for barcoding

## The primer

The preferred metazoan barcoding region is close to the beginning of the gene coding mitochondrial cytochrome C oxidase (COI). The COI-3 primer set from Ivanova et al. (2007) is used to amplify 650bp of the COI from fish. This primer cocktail VF2, FishF2, FishR2, FR1d (Ratio 1:1:1:1) is basically the M13 tailed COI primer from Ward et al. (2005). The two forward primer are M13F tailed, whereas the two reverse primer are M13R tailed to facilitate

sequencing and get less missing data as the first 50bp are often discarded due to low read quality. The Ivanova et al. (2007) primer anneal at the same position as the metazoan Folmer primer (Folmer et al. 1994), which fueled the barcode of life project 30 years ago. (See annealing position in figure 8 for LCO1490).

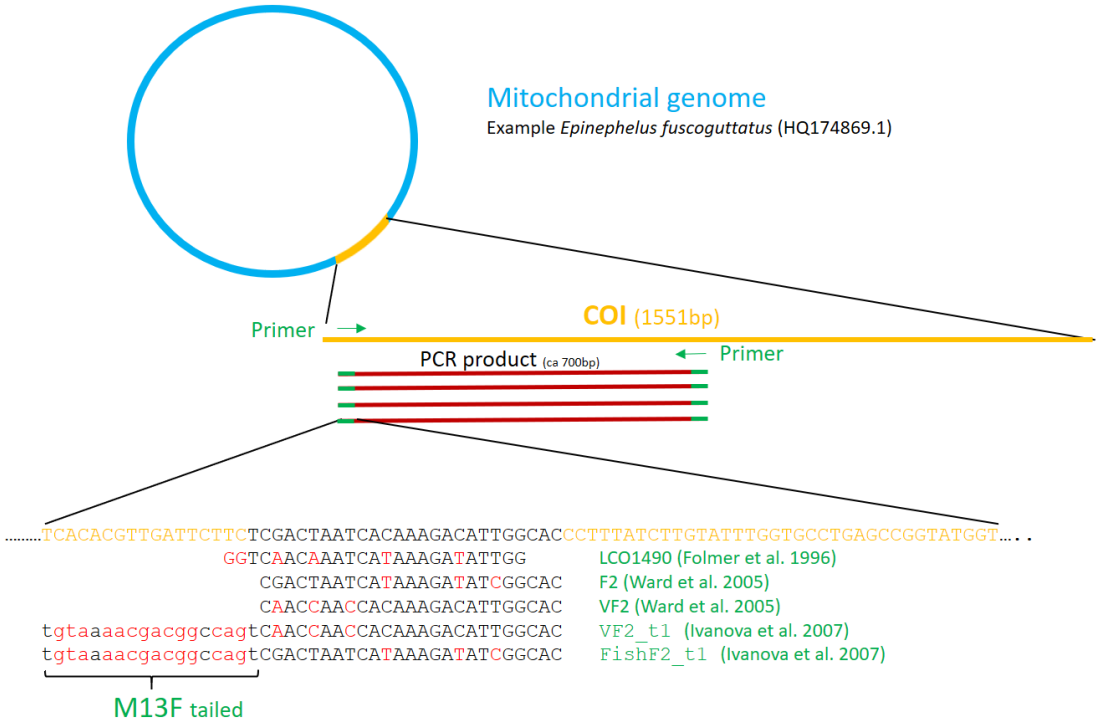


Figure 8: COI primer binding sites with a detailed example for the mismatches (red color) for all forward primer mentioned in the manual. The final tailed PCR product will be between 680-750bp due to primer selection (M13 tailing adds 35bp) and species depended indels. COI forward primer shown as example.

## Material primer dilution from lyophilized primer stock and working solution

- Lyophilized primer stock (tab. 4)
- Nucleic free water (Molecular Biology Grade Water, not distilled water from the lab)
- 1.5 ml reaction tubes (eppendorfs or "epps")
- 20 – 200 µl pipette filter tips a
- 1000 µl filter tips
- Pipettes
- UV-hood.

Table 4: COI primer cocktail from Ivanova et al. (2007) used for PCR and sequencing. M13F sequence red fonts, M13 reverse sequence blue fonts.

| Name | Sequence |
|---|---|
| VF2_t1 | tgtaaaacgacggccagtcaaccaaccacaaagacattggcac |
| FishF2_t1 | tgtaaaacgacggccagtcgactaatcataaagatatcggcac |
| FishR2 | caggaaacagctatgacacttcagggtgaccgaagaatcagaa |
| FR1d | caggaaacagctatgacacctcagggtgtccgaaraaycaraa |
| M13F | tgtaaaacgacggccagt |
| M13R | caggaaacagctatgac |

### Protocol primer dilution from lyophilized primer stock

Lyophilized primer are more stable than dissolved primer; therefore dry primer are preferred for long term storage and shipment.

1) Wipe the workplace with alcohol (e.g. Incidin) or clean soap water.
2) Expose the hood with 15min UV light to reduce possible sources of foreign DNA. Put empty tubes, tips, pipette and water inside the hood
3) While waiting for decontamination spin down the primer pellet to the bottom of the tube (max speed for 10s).
4) Check the manufacturer's instruction on the volume of water (alternatively TE buffer) added.
5) Pipette the given volume of nucleic free water into the primer stock. (e.g for M13F pipette 327 µl (different value for each new batch) of nucleic free water into the provided primer stock tube to get a 100 pmol/µl stock solution, i.e 32.7nM (nano mole) of primer are dissolved in 327µL of $H_2O$).
6) Vortex.
7) Centrifuge at maximum speed for 10 seconds.

### Protocol preparing the primer working solution

Use the stock solution to prepare a 1:10 (10 pmol/µl) working solution for each primer

1) Pipette 450 µl nucleic free water (or TE buffer) in a 0.5ml or 1.5ml reaction tube
2) Pipette 50 µl of the primer stock into the reaction tube.
3) Vortex thoroughly.
4) Spin down at maximum speed for 10 seconds using a table top centrifuge
5) Optional: Aliquot 100 µl of the working stock in four reaction tubes.
6) Optional: Pool both forward primer VF2 and FishF2 (1:1) in one forward primer tube.

Optional: Pool both reverse primer FishR2 and FR1d (1:1) in one reverse primer tube. Freeze aliquots (-20°C) for storage. The working solution can be stored in the fridge for several weeks.

## PCR reaction

Mostly PCR is accomplished using conventional Taq polymerases in a 2x master mix (buffer + $MgCl_2$ + dNTPs + Taq) for easy pipetting and having all ingredients with low fluctuations of concentration. Hence conventional Taq is unstable at room temperatures and on some occasions such as air travel, uncooled transport is the preferred shipment for budget reasons. "Hot Start" Taq DNA polymerases usually cope better at such suboptimal shipment conditions because they develop their enzymatic activity only after initial heating to 95°C. Though complex reaction mixtures tend to react in warm environment, thus with heat protected polymerases a PCR master mix is not anymore the best choice if cooling cannot be assured during transport or lab supply chains are weak. Loss of enzymatic activity is much reduced if using separately packed single components. Therefore, we use a kit providing separate vials of the single components dNTP's, reaction buffer and polymerase. Antibody mediated hot start polymerase has import restrictions in some countries due to its genetically engineered modifications. In conclusion to facilitate international transport at ambient conditions, we used a chemically inhibited Hot-Start polymerase with a single component kit. The here used onTaq polymerase (Roboklon EK2713-01) is such a chemically inhibited hot start enzyme, which is

available as separate components and was successfully transported from Bremen, Germany to Mombasa, Kenya without cooling and successfully used in a warm, 28°C working environment. In addition to the described shipment advantages, the (hot) start reduces unspecific primer binding and miss-amplification, thus giving higher specificity (sharp bands) and enabling pipetting in a warm environment without the use of ice or PCR coolers (such as Eppendorf PCR Cooler 3881000015). Despite tolerating non cooled handling, pipetting should be conducted quickly in a warm environment, in particular if working with small volumes due to evaporation issues with uncooled reaction tubes. If PCR coolers are available, this will always be the preferred option.
As a final remark for lab organizers: In areas with power cuts it might be a good investment providing an Uninterruptible Power Supply (UPS) and check for planned power interruptions in advance of setting up PCR reactions (e.g. using the *Power Outage Monitor* app on your mobile phone).

## Material for the PCR reaction

Based on the ON Taq kit (Roboklon EK2713-01)

- sterile water (PCR reaction)
- Buffer C (red colored for easy gel loading, provides 1.5mM $MgCl_2$ in the final reaction)
- $MgCl_2$ (Optional for possible optimization experiments)
- dNTPs
- ON Taq (polymerase)
- Forward primer mix (1:1 VF2, FishF2)
- Reverse primer mix (1:1 FishR2, FR1d)
- gDNA template in a dilution series (1:10, 1:20, 1:50, 1:100)
- Filter tips
- 200µl Reaction tubes, stripes or a 96 well plate with foil for sealing
- Pipettes
- UV Hood
- Thermocycler

## Protocol for the PCR reaction

1) Wipe the workplace including the UV hood with alcohol (e.g. Incidin) or clean soap water.
2) Expose the hood with 15min UV light to reduce possible sources of foreign DNA.
3) Program a cycler to have the device ready directly after pipetting. For the VF2, FishF2, FishR2, FR1d Primer cocktail use the following temperature scheme shown in figure 9.

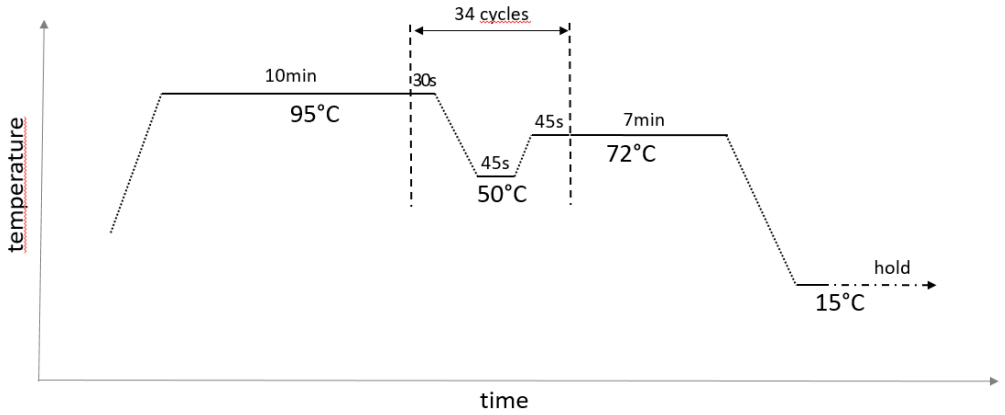


Figure 9: PCR cycling scheme. The long 10min initial hot temperature is mandatory to release the chemically inhibited onTaq. Other polymerases would require shorter initial heating as there the goal is to denature double stranded DNA while preserve the polymerase from excessive heat degradation. Annealing temperature for the applied COI-3 primer set might be varied between 48°C to 58°C. Avoid 4°C for the final hold as condensation water will corrode the heating block, while not needed for PCR products. Double stranded DNA in PCR buffer is very stable and can be well kept at 15°C until transferred to a fridge.

4) Take the ingredients to the UV Hood, but leave the polymerase in the freezer.
5) Label each 200µl reaction tube with a unique identifier (e.g. for five samples label the five samples to be sequenced plus the positive control and the negative control: A00, A01, A02, A03, A04, +, -)
6) Create a table with all necessary components in your lab book. Calculate one reaction more as an excess of master mix is accounted for pipetting loss.

Table 5: Pipetting scheme of the PCR master mix in the order of addition to the tube. Example data in green fonts for 5 samples, thus 7 reactions including the positive and negative controls. (In the example with grey fonts we calculated generously for 8rxn to allow for pipetting loss.)

| **Component** | **Concentration Single reaction** | **Stock** | Volume per reaction [µl] | Volume per [8] reactions [µl] |
|---|---|---|---|---|
| **$H_2O$** | | | 19.88 | 159,04 |
| **(10X) Buffer C** contains 15mM $MgCl_2$ | 1x | 10x | 2.5 | 20 |
| **dNTPs** | 0.2mM each dNTP | 4x10mM | 0.5 | 4 |
| **$Mgcl_2$** | 1.5mM | 25mM | 1.5 | 12 |
| **Pooled forward primer** | 0.1-0.5µM | 10 µM | 0.5 | 4 |
| **Pooled reverse primer** | 0.1-0.5µM | 10 µM | 0.5 | 4 |
| **Other** (BSA, 10% trehalose, …) | - | - | - | - |
| **onTaq Polymerase** | 0.62 units | 2.5 U/µl | 0.12 | 0,96 (use 1µl) |
| **Template gDNA** | <0.25µg/25µl | | 1 | Not included |
| **Total Volume** | Transfer 24µl of the master mix into each reaction tube | | **25 µl** = final volume per reaction inc. gDNA | ∑ 192 |

7) Pipette the reagents from the last column in the order of appearance in a 1.5ml tube. It is a good habit to take the polymerase only briefly out of the freezer when needed.

8) Vortex. # Thorough mixing is important because polymerase is stored in glycerin and thus congregate at the bottom of the master mix tube.
9) Spin or shake down droplets from the lid or tube walls. If using plates a salad spinner might be adopted for plate centrifugation (Morán and Galindo 2011).
10) Distribute the single reactions, use a multipipette for plates. In the example (table 5) 24µl are distributed in 7 tubes.
11) Add the gDNA template to the reactions (usually 1-2 µl of a 1:10, 1:20 and 1: 100 dilutions? of the gDNA extraction) outside the UV hood. Stay focused if working with plates, it is easy to mess up! For the negative control add the same amount of liquid from the DNA extraction without tissue (1 µl in the example of table 5).
12) Close the tubes or seal the plate.
13) Take notes of your labeling (figure 10).
14) Move to the post PCR room with cyclers.
15) Place the tubes or plate in the prepared thermocycler, close the lid carefully and press run.
16) After completion of the run proceed with downstream applications such as agarose gel or store at 4°C for short term or -20°C for storage above one week.

Figure10: Keep from records during your lab activities either using an electronic lab book as shown here, or paper lab book.

## Agarose gel

Gel electrophoresis separates DNA fragments by size in a solid support medium such as an agarose gel. Samples (DNA) are pipetted into the sample wells, followed by the application of an electric current (50-150V) which causes the negatively-charged DNA to migrate (electrophorese) towards the anodal (+) end. The rate of migration is proportional to size: smaller fragments move more quickly and wind up at the bottom of the gel. There are two Tris based agarose buffer available with either boric acid (TBE; Tris, Boric acid, EDTA) or acetic acid (TAE, Tris, Acetic acid, EDTA). Despite running behavior (speed) and resolution is better with TBE for the small target size of 750bp, we use TAE because of reduced health issues and because with TAE 50-fold stock solutions are easily possible whereas TBE stock solutions have to be prepared as 5x concentrate because precipitation takes place at higher concentrations such as 10x. TAE also facilitates recovery from gel if a clean product is needed from gel extraction. See Heery et al. (1990) for a simple DNA recovery from gel slices without a kit.

## Material for the agarose gel

- 1g of agarose gel.
- 100ml of 1X TAE/TBE buffer. Use always the same buffer for gel and tray.
- Stain G (Serva, HS 38229000), fluorescence exication maximum with DNA at ca. 450 nm
- DNA Ladder (Thermo, Gene ruler 100bp plus: SM0322)
- 50x TAE (weigh out 242 grams of Tris-base (MW = 121.14 g/mol) and dissolve in approximately 700 milliliters of deionized water. Carefully add 57.1 milliliters of 100 % glacial acid (= concentrated acetic acid) and 100 milliliters of 0.5 M EDTA (pH 8.0) and adjust the solution to a final volume of 1 liter. The pH of this buffer is not adjusted and should be about 8.5). Store stock solution at room temperature.
  See also: dx.doi.org/10.17504/protocols.io.gtvbwn6
- 1x TAE (to 20ml 50xTAE fill up to 1000 ml with deionized water)
- Gel system such as Mupid-exu submarine electrophoresis system
- Parafilm
- TAE Buffer
- Microwave oven
- 100ml glass beaker
- Gel stain
- Gel documentation

## Protocol for the agarose gel run

1) Weigh 1 gram of agarose powder into 100mL of electrophoresis TAE buffer.

Table 6: Weigh of agarose needed for various concentrations in 100ml TAE buffer to be run at 4-10 V/cm. The lower the gel concentration, the faster DNA runs. The preferred agarose concentration for 700bp COI is 1% (Bold)

| Agarose weight (g) | Agarose gel percentage | Range of effective separation [bp] | Approximate positions of dye in TAE [bp] | |
|---|---|---|---|---|
| | | | Bromophenol blue | Xylene |
| 0.8 | 0.8% | 800-10000 | 530 | 6500 |
| **1.0** | **1.0%** | **400-8000** | **370** | **4160** |
| 1.5 | 1.5% | 200-3000 | 190 | 1840 |
| 2.0 | 2.0% | 100-2000 | 120 | 1040 |
| 3.0 | 3.0% | 25-1000 | 60 | 460 |

2) Place in the microwave oven and heat on high power for one minute.
3) Remove carefully as any microwaved solution may become superheated and foam over when agitated. Gently swirl to re-suspend any agarose particles.
4) Reheat on high power using 15-20 second intervals or until the solution comes to a boil, and dissolving is complete.
5) Remove carefully and allow to cool 50-60°C at room temperature (depending on volume, e.g. 3-10 minutes).
6) Add Stain G (final concentration 0.02 ug/ml) to facilitate visualization of DNA after electrophoresis and gently swirl to homogenize, before pouring the solution into the gel tray with gel combs in place allowed to solidify at room temperature.

7) Note: Remove any air bubbles formed at the comb with a pipette-tip before the gel finally solidifies.
8) After the gel has solidified, the comb is removed, taking care not to rip the bottom of the wells.
9) The gel, still in plastic tray, is inserted horizontally into the electrophoresis chamber and is covered with buffer.
10) Samples containing DNA mixed with loading buffer are then pipetted into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied (We use 5 µl of the PCR product in the well and 6 µl DNA ladder; with our tray 100 volts for 25min).
11) The electric current can be confirmed by observing bubbles coming off the cathode.
12) DNA will migrate towards the anode (+), which is usually colored red, and taking the negative charge.
13) The distance DNA has migrated in the gel can be judged by visually monitoring migration of the tracking dyes like bromophenol blue and xylene dyes (see table 6).

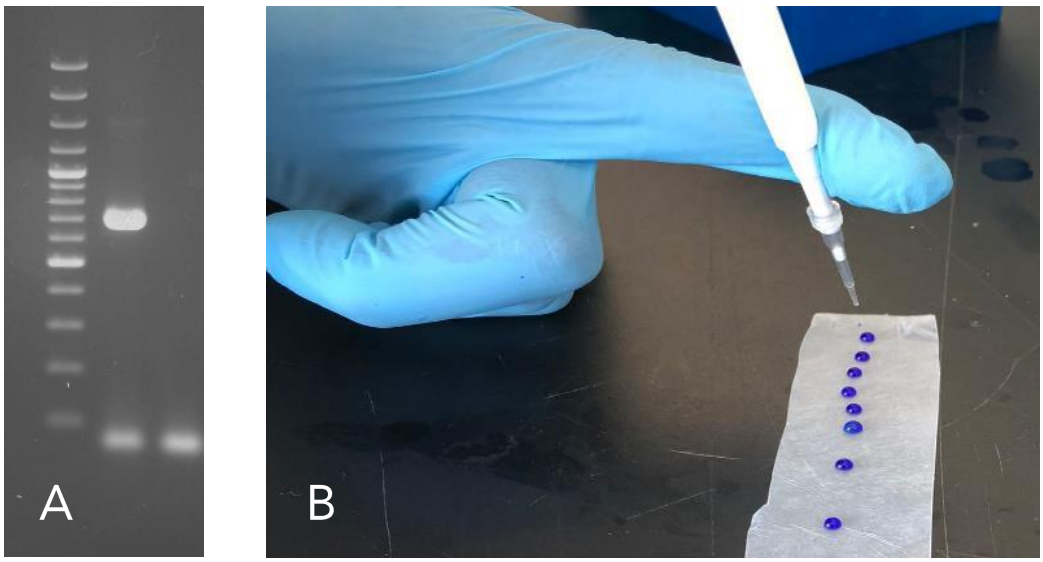


Figure 11 panel A: DNA standard for size control and quantification. If prepared as described in the Generuler protocol using 6µl ladder results in 80ng DNA at the 500bp as well as in the 1000bp band. By comparing the brightness of your PCR product you get a good guesstimate (here >100ng) for the PCR product quantification in addition to the size control (700bp). Panel B: Mixing PCR products with loading dye on a parafilm.

## PCR trouble shooting

Ideally, when you look at the agarose gel with your PCR products you will see a nice, bright, tight band of the correct size from each reaction and the lane with your negative control should be clean. Unfortunately, this is not always the case - there are many other possibilities!!! (see e.g.: Palumbi et al. 2002). Below we list a few possible outcomes with explanations and possible handling instructions.

**No PCR product**

a) Empty gel without DNA marker (ladder) and no traces of DNA visible in the wells.

*You may have forgot the staining solution or use the wrong combination of wavelength and stain for visualization. First confirm the functioning of the light source and camera of the gel documentation system. In case staining was forgotten you may rescue this gel provisional by placing the TAE gel in a TAE buffer bath with excess stain for 10min, or repeat the run using a gel that includes staining (preferred). Also excess of runtime may lead to bands running out of field of view. Keep in mind that the stain migrates in the opposite direction (towards the cathode) and thus narrows the detection size as well.*

b) No DNA marker (ladder), but (some) wells luminesce brightly (contain DNA).
*Ensure voltage, thus confirm little bubbles at the cathode of the gel apparatus when switched on.*

c) DNA marker (ladder) is visible, but no PCR product including no positive control.
*Detective work: Check the pipetting scheme. You need to confirm if you forgot any ingredients in the PCR set up such as dNTPs, gDNA template or polymerase. Did the cycler run? Did you include a positive control? Is the selected primer working on your organisms including the positive control? Did you use the correct template concentration? Here are some suggestions:*
   - *Try it again.*
   - *Recalculate the pipetting scheme. If different try again with the corrected scheme.*
   - *Try it again with a new kit (polymerase).*
   - *Get your positive control working, thus repeat the PCR conditions where you determined this template as valid positive control and repeat with exactly the same conditions and primer. In parallel repeat the positive control with the new primer from the failed run (pipette one master mix without primer, mix thoroughly and divide into two tubes to add the different primer. Use one positive control and negative control in each of the two assays).*

**No PCR product for the targets only**. DNA marker (ladder) is visible and PCR was successful for the positive control.

a) Low gDNA concentration or degraded gDNA template
*Check gDNA integrity on a gel or do at least a photometric quantification of your undiluted template. If gDNA cannot be detected repeat your extraction or revisit your collection and fixation method. Sometimes high end polymerases or whole genome amplification in advance of the PCR can rescue the situation.*

b) PCR Inhibition
*If presence of template DNA is confirmed (see above), try other gDNA concentrations by using a dilution series of your gDNA as template (1:10 up to 1:100). Diluting reduces the effect of co-eluted inhibiting substances: It is possible that something (e.g. melanin) in your DNA extraction is interfering with the PCR reaction. This can be determined by adding the well working positive control gDNA into the target PCR (two gDNA templates in one reaction. When this reaction is still not amplified with introduced positive control you proved inhibition. -> dilute with sterile water, add 10% trehalose or BSA (Silva et al. 2020) or clean up your target gDNA using e.g. a column based kit.*

*c)* Poor matching of primer
   - *Try lowering the annealing temperature. Your target sequence may differ from the expected sequence motive.*

- *Try 5 or 10 cycles at a very low annealing and extension temperature (42°C for instance), then another 30 cycles at higher temperatures. Attention, you may amplify the wrong product.*
- *Use PCR enhancers such as BSA (Nagai et al. 1998).*
- *Re-amplify the PCR product by using 1µl from an at least 1:10 diluted PCR reaction with the M13 primer set. Thus you use the first PCR as template in a second reaction similar to a nested PCR.*

d) Too much template DNA
Bright bands in the gel wells. PCR successful for positive control only and DNA marker (ladder) is well resolved and visible, sometimes combined with weak target bands. Either you see your excess template here, or the surplus of template prevented reaching the end of an amplicon during the elongation phase (polymerase is very fast). In each cycle a very long product was synthesized which is now trapped in the well. Very large DNA fragments do not migrate.
- *Try less gDNA.*

**Weak PCR products**. DNA marker (ladder) is visible and PCR was successful for the positive control.
Sequencing does not always need perfect amplicons thus maybe no action is needed and you proceed to sequencing as usual. However, there might be solutions to improve your results depending on the reason. Check your gDNA on a gel to detect degradation.

a) Degraded or low quantity DNA
*Extract gDNA again from tissue or revise your collection and fixation method. You may try adding a few PCR cycles but you may also introduce errors. Usually 35cycles are the maximum number, but sometimes up to 40 cycles were successfully used.*

b) Suboptimal $MgCl_2$ concentration
*Try varying $MgCl_2$ concentrations with a dilution series to determine which concentration results in the best bands. Hence optimization is usually not needed because sequencing works well with suboptimal products. The recommended range of $MgCl_2$ concentration is 1-4 mM. 1.5 mM is used in most cases and therefore provided in many complete buffers. If the gDNA samples contain EDTA or other chelators, the $MgCl_2$ concentration in the reaction mixture should be raised.*

Table 7: $MgCl_2$ pipetting volumes for one 25µl reaction mix (final volume) to adjust the $MgCl_2$ concentration

| Final $MgCl_2$ concentration in the PCR | **[mM]** | **1.0** | **1.5** | **2.0** | **3.0** | **4.0** |
|---|---|---|---|---|---|---|
| Volume of 25mM $MgCl_2$ | **[µl]** | 1.0 | 1.5 | 2.0 | 3.0 | 4.0 |
| Volume of 25mM $MgCl_2$ if 1.5mM is present in provided buffer | **[µl]** | n.a. | 0.0 | 0.5 | 1.5 | 2.5 |

c) Inhibition
*Use PCR enhancers such as BSA or other (Nagai, Yoshida et al. 1998)*

Smearing of PCR product, primer dimer or multiple bands
- *Try slightly higher annealing temperature to gain specificity, or use a touchdown protocol where you start with a high annealing temp (such as 60°C) and go down each cycle for 0.5 - 1°C until the desired annealing temperature is reached. 15-20 cycles are conducted on the low temperature.*
- *Try other primer or design new.*

**Bands in the blank**
- *Add a second negative control to exclude issues with the extraction.*

- *Change your PCR components, water is usually a good place to start, last the polymerase. Repeat your PCR until the negative control is negative again. Do not use the PCR products of the target samples. Often you will need to set up a new primer working solution from the stock or to replace the whole kit. Work in a clean environment, thus set up your reactions far away from PCR amplification areas, preferably in a UV hood at the Pre-PCR lab.*

## PCR Clean up

Enzymatic PCR-cleanup inactivates the remaining primer and unused dNTPs from a PCR reaction: Exonuclease I cleaves single stranded DNA (such as left over PCR primer) without interfering with double stranded DNA (PCR product). Alkaline Phosphatase dephosphorylates the remaining dNTP, so they do not interfere with the Sanger sequencing reaction such as the BigDye™ Terminator kit. This PCR clean up method enables direct downstream applications, such as Sanger sequencing. The two enzymes are added directly to the PCR reaction after thermal cycling, without changing the buffer or using additional additives. Both enzymes suggested below were successfully transported for 48h without cooling.

### Material for PCR Clean up

- Exonuclease I (Roboklon E1150-0)
- Polar Alkaline Bacterial Phosphatase (Roboklon E1027-01)
- 25µl PCR product just after amplification
- Thermocycler

### Protocol for PCR Clean up

1) Count the number of clean up reactions needed for PCR clean up. Calculate 0.5 µl (10 U) Exonuclease I per reaction and 1 µl (5 U) Polar-BAP per reaction.
2) Pipette both enzymes together (For example to prepare five reactions: 5 x 0.5µl = 2.5µl Exonuclease + 5 x 1µl = 5µl Alkaline Bacterial Phosphatase to a total volume of 7.5µl ExoBAP.
3) Add 1.5µl ExoBAP to each 25µl PCR product.
4) Place in a cycler. # Usually an ExoBAP or ExoSAP protocol is stored somewhere:
    - Incubate for 15 min at 37°C
    - Heat inactivation for 15 min at 80°C
    - Hold at 15°C
5) Up to 5 µl may be used directly for sequencing without any further purification. It is recommended to use PCR devoid of any non-specific products. Both enzymes are known to work in a variety of different buffer systems, thus no dedicated buffers are required but directly added to the PCR mix.

Send to sequencing using two reactions from each PCR, one including the M13F primer only and the second with the M13R primer only.

# References

Bajorath, J., W. Saenger, and G. P. Pal. 1988. Autolysis and inhibition of proteinase K, a subtilisin-related serine proteinase isolated from the fungus *Tritirachium album* Limber. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 954:176-182.

Darlene, F. O., and C. Phee-Kheng. 2013. Hot water immersion as a treatment for stonefish sting: A case report. Malays Fam Physician 8(1):28-32.

Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3.

Ghilardi, M. 2022. MorFishJ: A software package for fish traditional morphometrics. https://doi.org/10.5281/zenodo.7275017.

Heery, D., F. Gannon, and R. Powell. 1990. A simple method for subcloning DNA fragments from gel slices. Trends in Genetics 6:173.

Ivanova, N. V., J. R. Dewaard, and P. D. N. Herbert. 2006. An inexpensive, automation-friendly protocol for recovering high-quality DNA. Molecular Ecology Notes 6(4):998-1002.

Ivanova, N. V., T. S. Zemlak, R. H. Hanner, and P. D. N. Hebert. 2007. Universal primer cocktails for fish DNA barcoding. Molecular Ecology Notes 7(4):544-548.

Kilpatrick, C. W. 2002. Noncryogenic Preservation of Mammalian Tissues for DNA Extraction: An Assessment of Storage Methods. Biochemical Genetics 40(1):53-62.

Morán, P., and J. Galindo. 2011. A practical home-made microcentrifuge for teaching purposes. Biochemistry and Molecular Biology Education 39(4):298-299.

Nagai, M., A. Yoshida, and N. Sato. 1998. Additive effects of bovine serum albumin, dithiothreitol and glycerolon PCR. IUBMB Life 44(1):157-163.

Nagy, Z. T. 2010. A hands-on overview of tissue preservation methods for molecular genetic analyses. Organisms Diversity & Evolution 10(1):91-105.

Nilsson, M., H. De Maeyer, and M. Allen. 2022. Evaluation of Different Cleaning Strategies for Removal of Contaminating DNA Molecules. Genes 13(1).

Palumbi, S. R., and coauthors. 2002. The simple fool's guide to PCR. Dept. of Zoology and Kewalo Marine Laboratory, University of Hawaii, Honolulu, HI :.

Ratnasingham, S., and P. D. N. Hebert. 2007. bold: The Barcode of Life Data System (http://www.barcodinglife.org). Molecular Ecology Notes 7(3):355-364.

Robertson, K. M., J. Minich, A. J. Bowman, and P. A. Morin. 2013. A thin soup: extraction and amplification of DNA from DMSO and ethanol used as preservative for cetacean tissue samples. Conservation Genetics Resources 5(4):929-933.

Rodríguez‐Ezpeleta, N., I. Mendibil, P. Alvarez, and U. Cotano. 2013. Effect of fish sampling and tissue storage conditions in DNA quality: considerations for genomic studies.

Sharpe, A., and coauthors. 2020. DESS deconstructed: Is EDTA solely responsible for protection of high molecular weight DNA in this common tissue preservative? PLoS ONE 15(8):e0237356.

Silva, A. J., M. Kawalek, D. M. Williams-Hill, and R. S. Hellberg. 2020. PCR Cloning Combined With DNA Barcoding Enables Partial Identification of Fish Species in a Mixed-Species Product. Frontiers in Ecology and Evolution 8.

Steinke, D., and R. Hanner. 2011. The FISH-BOL collaborators' protocol. Mitochondrial DNA 22(sup1):10-14.

Steinke, D., R. Hanner, and P. D. N. Hebert. 2009. Rapid high-quality imaging of fishes using a flat-bed scanner. Ichthyological Research 56(2):210-211.

Ward, R. D., T. S. Zemlak, B. H. Innes, P. R. Last, and P. D. N. Hebert. 2005. DNA barcoding Australia's fish species. Philosophical transactions of the Royal Society of London. Series B, Biological sciences (0962-8436 (Print)).

## Acknowledgement

We thank the Volkswagen Foundation for funding the *workshop Fish Barcoding and Functional Ecology: Monitoring the Status of Marine Coastal Ecosystems* where this booklet resulted. We are also grateful to the Roboklon company who supported this work by providing consumables and technical advice. The workshop was enabled by a lot of support by KMFRI as well as ZMT staff. In particular we like to thank Albert Ositu, Andrea Daschner, Erick Okuku, Erick Okenagwa, Heike Holtkamp, Janine Reinhard, Joel Gatagwu, Melckzedeck Osore, Moses Kinyua, Sonja Peters and Timo Schumann.

Figure 12: Most of the authors at the integrated symposium during the workshop at KMFRI in October 2022 ☺

ZMT LEIBNIZ CENTER for Tropical Marine Research

# Protocols Series No. 1

https://doi.org/10.21244/zmt.2023.002
May, 2023

**Authors** (in alphabetical order of surnames)**:** Achim Meyer, Anissa Volanandiana, Cretus Joseph Mtonga, Debora Benjamen, Diana Karan, Erica Tovela, Humphrey Mahudi, Janine Baxter, John Kochey Kipyegon, Kennedy Oduor, Levy Michael Otwoma, Liban Isse Farah, Manda Kambikambi, Mattia Ghilardi, Mpilonhle Nyawo, Nathan Mrombo, Oscar Puebla, Said Mgeleka, Samuel K.K. Amponsah, Segun Olayinka Oladipo, Sonia Bejarano, Teresia Wacira, Tsiaranto Fanoro and Valarie Silali

**All photos** from the authors except figure 6 (Tom Vierus)

**Contact:** achim.meyer@leibniz-zmt.de

Published by

**Leibniz-Zentrum für Marine Tropenforschung (ZMT) GmbH**

**Leibniz Centre for Tropical Marine Research (ZMT)**

Fahrenheitstr. 6
28359 Bremen
Germany
Tel: +49 421 23800 21
Fax: +49 421 23800 30
Email: contact@leibniz-zmt.de
www.leibniz-zmt.de