Marine Invasive Species Early Detection: LABORATORY MANUAL

DEVELOPED by OBIS and USP for the PacMAN project

EMERGENCY PROCEDURES

1. Be familiar with location of fire blankets and extinguishers. To be used only in the event of localized minor fires.
2. In the event of major fire and/or the sounding of alarm, all participants should move to the assembly point outside of the building. Move rapidly, calmly via stairs if necessary –DO NOT USE A LIFT. Do not leave the assembly point until your name has been checked off the roll
3. Be familiar with the location and use of safety features such as eyewash and showers. Use immediately if needed. If in doubt, use water.
4. All accidents or spillages must be reported to trainers in charge.

Disability Disclosure

Any participant with a mental or physical health problem which may affect performance during the course activities is encouraged to consult with the trainers. The provision of this information is voluntary. However, it is important that you make known any health conditions that may affect your safety or the safety of others.

Laboratory Rules

1. Only necessary articles may be brought into the laboratory. Coats, bags etc. must be left outside.
2. A laboratory coat must be worn which covers the knees when you are seated, and shoes which protect the feet against acids and broken glasses.
3. On no account must any apparatus be placed in the mouth. Labels must not be licked. Smoking, sweets and food generally are FORBIDDEN.
4. Any accident e.g. spilling of samples or personal injury, however slight, must be reported to a demonstrator at once.
5. All samples must be labelled with their name and description

6. Return communal apparatus or reagents to their places as soon as you have finished.
7. Turn off water taps and lights when not actually using them; turn down burners when not being used. Turn off all water and electricity before leaving the laboratory.
8. At the end of the day, clean your bench of all equipment into containers provided.
9. Before leaving the laboratory, disinfect and wash hands. The same procedure must be followed IMMEDIATELY in the case of contamination or possible contamination of hands.
10. All participants must wear gloves at all times when dealing with DNA work
11. Follow all instructions implicitly and adhere rigidly to the technique you are taught. This will ensure your own safety, the safety of others and the reliability of your work.
12. Hair that is shoulder length or longer, must be tied back. This is to prevent possible contact with DNA samples or open flames on Bunsen burners.

Regard all materials as potentially infective and dangerous, and treat them accordingly.

Some Hazards and Precautions in DNA Technology

1. Aerosols

Whenever a liquid surface is broken, droplets are produces. The larger droplets fall but the small ones evaporate and may generate infectious air-borne particles if the liquid contains micro-organism. The conditions that lead to aerosols can persist for long periods and they can spread over a wide area exposing other workers in the vicinity to the danger of infection by inhalation.

2. Breakage and spillage

Breakage of DNA sample vessels or spillage of cultures can cause gross contamination of skin, clothing and work surfaces, and the generation of infectious aerosols. An infection may then be acquired through existing skin lesions, by transfer from the hands to the mouth, through the eyes, or by inhalation.

3. Ingestion

It can be appreciated that there should be no consuming of food, smoking, or pipetting by mouth in the microbiological laboratory as these activities can result in infection by ingestion.

4. Wounding and self-inoculation

Cuts from contaminated glassware and puncture due to faulty technique with syringes can lead to systemic infection.

5. Handling specimens

It is the unexpected as well as the expected organisms in pathological specimens which present risks. Consequently, the reception and opening of packages containing infectious specimen must be done by trained laboratory staff who are aware of the possibility of damage or leakage of containers in transit. Gloves must be worn when unpacking specimens. Unpacking must be done on a surface that is readily disinfected and the packing material placed in a plastic bag and autoclaved as minor in apparent leakage can occur during transport.

6. Pipetting

Infectious liquids must not be pipetted by mouth, primarily because of the risk of accidently sucking the liquid into the mouth. Also sucking of liquids into pipettes can be shown to generate an aerosol in the space above the liquid and cotton wool plugs may not be effective in containing an aerosol during pipetting. A manual pipetting aid must be used and it should be of a type that can be readily disinfected. Pipettes or pipette tips should not be discharged forcefully since this generates an aerosol. Discharge should take place as close as possible to the surface of the liquid, or the liquid allowed to run down the wall of the vessel. Used pipettes would be discarded in to a jar of disinfectant such that they may remain vertical and are completely immersed.

7. Centrifugation

The centrifuge is potentially an excellent generator of aerosols. To prevent aerosols, only capped centrifuge tubes should be used where possible. All centrifuge tubes must be inspected for flaws that might lead to leakage or collapse of the tubes during centrifugation. The breakage of a centrifuge tube containing infectious material is a serious accident that requires fumigation of the laboratory. If this has occurred, or is suspected to have occurred, do not open centrifuge. If using a fixed angle rotor and loose fitting caps, use volumes of liquid that do not lead to overflow under centrifugal force. Ensure that centrifuges are correctly balanced before operating and never open a centrifuge lid whilst the rotor is still in motion.

8. Pouring

Pouring one liquid into another will generate an aerosol. Pouring always contaminates the rim of the vessel that can transfer infective material to the outside of the vessel. However, where large volumes are involved, pouring may be a safer method than several transfers by pipette. Wherever possible pouring should be done in a biohazard cabinet.

9. Electrical apparatus

Ensure that electrical equipment is operated correctly. If in doubt of correct operating procedures consult the trainers in charge. Follow specific instructions for the safe use of particular items of equipment as detailed in the laboratory schedule.

10. Chemical Hazards

Some of the chemicals that are used in a molecular biology laboratory are extremely hazardous. Therefore it is essential that you read the appropriate chemical hazard information provided before use.

11. UV Trans illuminator

The UV light from the trans-illuminator is hazardous. Under no circumstance should you observe gels on a UV trans-illuminator with your naked eye. Use the eye protection provided. The UV radiation from the trans-illuminator will also burn exposed skin on prolonged exposure.

12. Accidents

All accidents must be reported to the staff member in charge.

Topic 1: Field Protocols for Monitoring, Deployment and Sampling Collections

1.1 Introduction

Invasive species are species that have been introduced to an area outside of their natural range and which cause substantial negative impacts on the environment they have become established in. This training course has been designed to build scientific capacities for marine invasive species monitoring through the use of well-established methods for the surveillance of biofouling, planulae level and micro-level (crytogenic-species of unknown origin) organisms in the marine environment.

Activities	P1	P2	P3	P4	P5	P6	P7	P8
Combined stakeholder meeting and first draft of communication pathway and management strategy								
Plate deployement and collection of old plates.								
Water and plankton collection and sample processing								
DNA extraction (eDNA), Sample preparations								
Library preparations and								
qPCR analysis								
Management plan recommendations								
Advisory meeting (FIST etc)								
Communication and Awareness (Alerts)								

1.2 Environmental Measurement

Environmental measurements provide the context for ecological analyses, and long-term monitoring of ecosystem state, as well as ecological conditions for MIAS.

Required Materials

- Digital YSI logger
- GPS logger
- Secchi disc or turbidity meter
- Digital camera
- Field data sheet

- Pen

- **Protocol**

  For sampling location at the jetty
  - Record GPS coordinates
  - Record water depth at location
  - Water salinity and temperature should be measured at least at 2.5 m intervals from surface water to bottom at each site
  - Measure also pH, dissolved oxygen and turbidity if possible
  - Fill in environmental data sheet
  - Take pictures of relevant conditions in the sampling locations (e.g. extensive biofouling)

Field sampling sheet: Environmental data

Port	Site	Description of site	Coordinates	Date of sampling	Total water depth	Measurement depth (m from surface)	Salinity (ppt)	Temperature (°C)	Dissolved Oxygen (mg/l)	pH	Turbidity
						0					
						2.5					
						5					
						7.5					
						0					
						2.5					
						5					
						7.5					
						0					
						2.5					
						5					
						7.5					
						0					
						2.5					
						5					
						7.5					

1.3 Water Sampling

Required Materials

- 1L sterilized water bottles (e.g. Nalgene™), marked for exact 1 liter level. (4-5x for each site)
- Sterile gloves
- Thermal box and ice for cooling samples
- Water-resistant marker/tape

- **Protocol**

  Preparation before using/re-using sample bottles,

  - Decontaminate by submerging in 10 percent bleach solution
  - Rinse thoroughly with distilled water (fill, cap, shake, and rinse; repeat at least three times), let dry
  - At the sampling site, rinse again with sample water three times (cap and shake) to remove any remaining bleach before collecting sample. This step requires a lot of care as any remaining bleach will degrade eDNA!
  - Collect three replicate 1 L surface water samples at site
    - Label with:
    - Date_ Location_SampleType_Depth_replicate
    - (e.g. 20211105_Suva_Site1_Water_0m_A)
  - Place in cooler for transport to lab
  - Fill in collection data sheet
  - At lab, either filter immediately (protocol 4.3.1) or place in +4 °C overnight for a maximum of 12 hours
  - Collect also an extra 1 litre of water at each site to be used in the processing of biomass samples.

Field sampling sheet: Sampling data

Port	Site	Date of Sampling (day, month, year)	Time ([hh]:[mm])	People sampling	Total water depth

Sample Type	Water			Plankton		Settlement plate		
				µm	µm			
Collected?								
Depth								
Amount								
Duration								
Method								
Pretreatment								
Storage								
Replication								

Comments

1.4 Settlement Plates

Required Materials

- 100mm diameter pipes x 4 lengths
- Polypropylene rope (0.5 cm diameter), approx. 22 m
- Short tubing (hard plastic, to place between PVC plates and rope)
- Zip ties
- Quarter inch rod
- Bricks
- In case of no suitable structure at the sampling sites, deploy on own buoys

- **Preparation:**
  - Cut PVC pipe in half lengthwise, to get two half-circle shapes (~plates)
  - Sand both sides of each PVC pipe briefly (few seconds, sanding paper 80)
  - Drill hole in the in the center of each plate (~0.5 cm diameter)
  - Place short piece of tube at each hole on the PVC pipes (prevents breaking the rope due to movement of the setup in the water).
  - Secure PVC pipes with knots secured with zip ties, so that there is 2m of rope between plates A and B, and 4 m between pipes B and C, and ample rope at each end.
    - This is depending on the depth of water at site, and the depth that the settlement plates will be deployed, the recommendation is at 1 m, 3 m and 5m water depth.
  - Tie a brick at the end of the rope

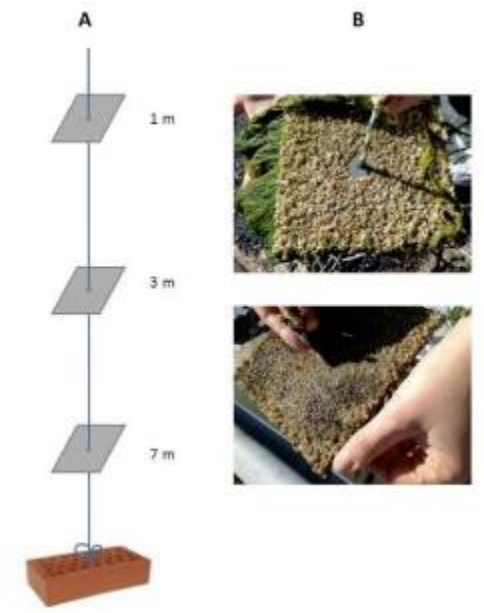


Figure 3. Suggested setup of settlement plates (Joint HELCOM/OSPAR guidelines). Diagram not to new standard placement lengths. Plates will be replaced by PVC pipe segments cut in half. Diagram not in proportions and should be adjusted according to peak low tide levels and site depths with lowest plate placed no more than 7 M deep and highest no more than 1 m from the water surface.

- **Deployment:**
  - ∇ Deploy duplicate setups at a location where they do not disturb port traffic
  - ∇ Check depth of water at site and adjust height of plates appropriately
  - ∇ Tie upper end of rope securely to a dock structure
  - ∇ Unit should remain upright and the rope should remain tight

Collection of settlement plates

Required materials:

- Single-use (sterile) plastic bags (20x20 cm) labelled for collection (Ziploc bags)
- Water-resistant marker
- Sterile gloves
- Cutters for the zip ties
- Thermal box and coolers for transportation
- New prepared settlement plates (full setup) for replacement

- **Collection:**
  - Retrieve plates after 2-3 months soak time
  - Pull on the dock/boat as carefully as possible
  - Place in individual plastic bags labelled with sample information
    - Date_Port_Location_SampleType_Depth_replicate
    - (e.g. 20211105_Suva_Site1_Plate_5m_A)
  - Place plastic bags in cooler for immediate transport to lab

1.5 Plankton Sampling

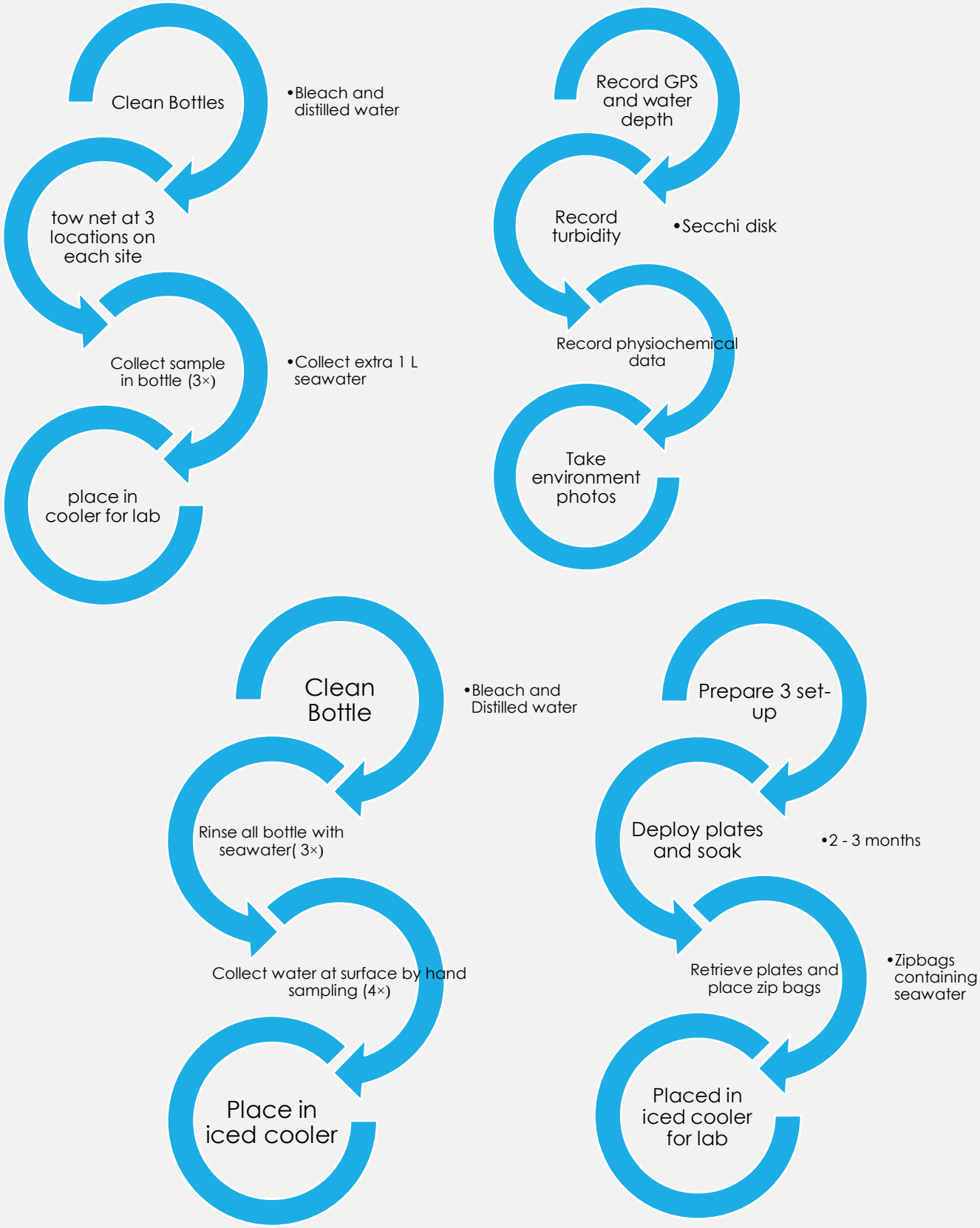
Required materials

- Plankton net with mesh sizes of 60 um and 280 um (what is available)
- Sterile 250-500 ml collection bottles for samples
- Marker
- Squeeze bottle for rinsing
- Sterile gloves

- **Protocol**
  - Tow Plankton net:
  - Drop net to 1 m from the bottom, and slowly bring back up (0.5-1 m/s)
    - Several tows may be needed to collect enough material for extraction at each site
    - Collect each sample from the tow in a clean sample bottle. If multiple tows are required, the sample can be concentrated by using the codend of the plankton net
    - If using different size classes these can also be combined if necessary
  - Pour collected material in collection bottle, marked with sample information
    - Date_Port_Location_SampleType_MeshSize_replicate
    - (e.g. 20211105_Suva_Site1_Plankton_200µm_A)
  - Place in cooler for transport to lab
  - Rinse codend of the plankton net three times with seawater at the collection site, and the next sampling site before new tow
  - In lab, centrifuge sample immediately, or place in +4 °C for a maximum of 12 hours

PacMAN

Summary for Field Protocols



Topic 2: Sample processing

All the following steps of the methods should be done in an area in the lab dedicated to eDNA work, to minimise contamination from other lab activities.

2.1 Processing of settlement plates

For one series of plates for eDNA:

Required materials

- Sterile gloves
- Sterilized tweezers
- Sterilized razor blades
- 50 ml sterile falcon tubes for sample collection
- Tube holder for falcon tubes (cleaned by submerging into bleach and rinsing with water).
- Mortar and pestle/homogenizer/blender for sample prep
  - e.g. kitchen blender

- **Protocol**
  - Wipe lab bench with >70% ethanol and 4.5% bleach
  - Set a sterilized kitchen foil on bench for any dropped material
  - Sterilize with ethanol and flame
  - With tweezers (or find another way here?), lift plate out of bag, and scrape biomass from both sides of the plate into falcon tube.
  - Homogenize biomass with additional site seawater on maximum speed in blender
  - Return to falcon tube
  - Label with sample label
  - Freeze at -20 C before DNA extraction (if not to processed the same day
  - For DNA extraction, measure 100-200 mg of homogenized and well-mixed biomass to a sample tube containing beads for bead beating.

For the second series of plates (for specimen sorting):

Required materials

- Squeeze bottle with clean seawater
- Waterproof Digital camera
- 75% ethanol
- Dissecting microscope
- Scalpel blades
- Waterproof labels and pen
- Notebook for notes and drawings
- Soft forceps
- Fine-haired paintbrush
- Petri dishes

- **Protocol**
  - Using clean seawater, rinse as much sediment as possible from the plate surface. Take a photograph of both sides of the whole plate with the plate label included.
  - Identify different morphotaxa on each plate surface and give them a number.
  - Take samples of each morphotaxa and store in 75 % ethanol after sorting each specimen to a phylum or the lowest taxonomic unit possible.
  - Keep your eyes open for any morphotaxa that may resemble any of the 12 species on the priority watchlist.
  - If time allows describe and photograph each morphotaxa.
  - It is desirable for all samples to have a label with as much information as possible about the living organism including its size, colour and texture.
  - Store each specimen for transfer to the USP Marine Collection

2.2 Filtering of water samples

Required materials

- Filtration setup 47 mm diameter (filtration cup, filter holder, collection Erlenmeyer)
- Vacuum pump and connecting tubing
- 0.45 µm filters 47 mm diameter (cellulose nitrate), autoclaved.

- Sterile 1 L deioinized water in a clean Nalgene bottle for preparing control sample.
- 4.5 % bleach for cleaning
- Sterilized tweezers
- Sterile 15 ml falcon tubes for filter collection
- Permanent marker
- Sterile small metal scissors (for example nail scissors)

- **Protocol**
  - Clean bench with >70% ethanol and 4.5% bleach before work
  - Wear sterile gloves at all times and try to minimize contamination with careful working methods.
  - Clean filtration system by submerging in 4.5 % bleach and rinsing thoroughly with deionized water between samples
  - Setup filtration system, and condition the filter with a small amount of deionized water
  - Keep 1 l bottle of deionized water or tube of DNA free water open during filtration to collect control sample
  - Record level of water in sample bottle, if not at 1 L mark
  - Pour sample water slowly on filter while keeping vacuum pump on.
  - If filter clogs, record amount of water remaining (total amount filtered)
  - Collect filter with sterile tweezers to falcon tube.
  - Label with the label on sample bottle

Topic 3: Laboratory molecular analyses protocols

3.1 DNA extraction (all sample types)

Required materials

- Sterile gloves
- DNA extraction kit (DNeasy Blood and Tissue Kit), containing extraction buffers and Proteinase K
  - Make sure that all preparation steps for the kit are done.
- 0.5 mm and 0.1 mm glass beads (BioSpec Products), ashed or sterilized and cleaned
- Sterile Eppendorf tubes (sterilized under UV if possible)
  - 2ml with screw cap and o-ring or suitable for bead beating
  - 1.5 ml eppendorfs
- Bead-beater
- DNAse free water
- Biosafety Cabinet will be used to conduct extractions when possible
- Centrifuge (for Eppendorf tubes)
- Vortex
- Heat block/bath
- Pipettes and DNAse free tips with filters (1000 µL, 200 µL, 100 µL, 10 µL)
- 100% molecular grade ethanol
- QBIT/Nanodrop

- **Protocol (https://www.protocols.io/view/mbari-environmental-dna-edna-extraction-using-qiag-xjufknw?step=4)**

  - Prior to extraction, 0.5 mm and 0.1 mm glass beads (BioSpec Products) need to be soaked in 4.5% bleach for 20 min, rinsed at least 3x with milliQ water and dried before autoclaving
  - Bead tubes: Distribute 0.25 g of each size glass bead into sterile 2.0-ml conical microcentrifuge tubes (with screw cap and o-ring). Autoclave tubes for 15 min at 121°C
  - Transfer sample (filter or biomass) to bead tubes with sterile forceps/spatula
    - 100 mg of tissue or filter
  - Add 720 µL Buffer ATL (Qiagen), and perform two bead-beating steps
    - Maximum speed for 45 sec, followed by incubation at 56 °C for 30 min
    - Repeat bead beating and incubation
  - Add 80 µl Proteinase K to each tube and incubate at 56 °C for a minimum of 2 hours, or overnight
  - After incubation, vortex tubes for 15 sec then centrifuge for 1 min at 4,000 x g

- Transfer 650-µL µl of supernatant to new 1.5-ml tubes then spin at 13,000 x g for 1 min
- After the final spin, transfer 600 µl of supernatant (avoiding any remaining glass beads) to a new 2-ml tube for the next steps
- Add 600 µl of Buffer AL to the sample, mix thoroughly by vortexing and add 600 µl of 100% ethanol and mix thoroughly by vortexing

It is essential that the sample, Buffer AL and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogenous solution. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples.

A white precipitate may form an addition of Buffer AL and ethanol. This precipitate does not interfere with the DNeasy procedure. Some tissue types (e.g., spleen, lung) may form a gelatinous lysate after addition of Buffer AL and ethanol. In this case, vigorously shaking or vortexing the preparation is recommended.

- Pipette 500 µL of lysate to spin column then centrifuge each time until the entire volume of lysate (1.8 mL) has passed through the spin column (can also be centrifuged). Centrifuge at ≥ 6000 × g (8000 rpm) for 1 min. Discard flow-through and collection tube
- Perform two 500-µL washes of Buffer AW1, centrifuge for 1 min at ≥ 6000 × g (8000 rpm). Discard flow-through and collection tube

- Perform two 500-µL washes of Buffer AW2, centrifuge for 3 min at 20000 × g (14000 rpm) to dry membrane. Discard flow-through and collection tube

It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20 000 × g (14000 rpm)

- Place the DNeasy Mini spin column in a clean 1.5 mL or 2 mL microcentrifuge tube, and pipette 50 µl of Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥ 6000 × g (8000 rpm) to elute. Elute in two 50-µl steps for a total of 100 µL extracted DNA

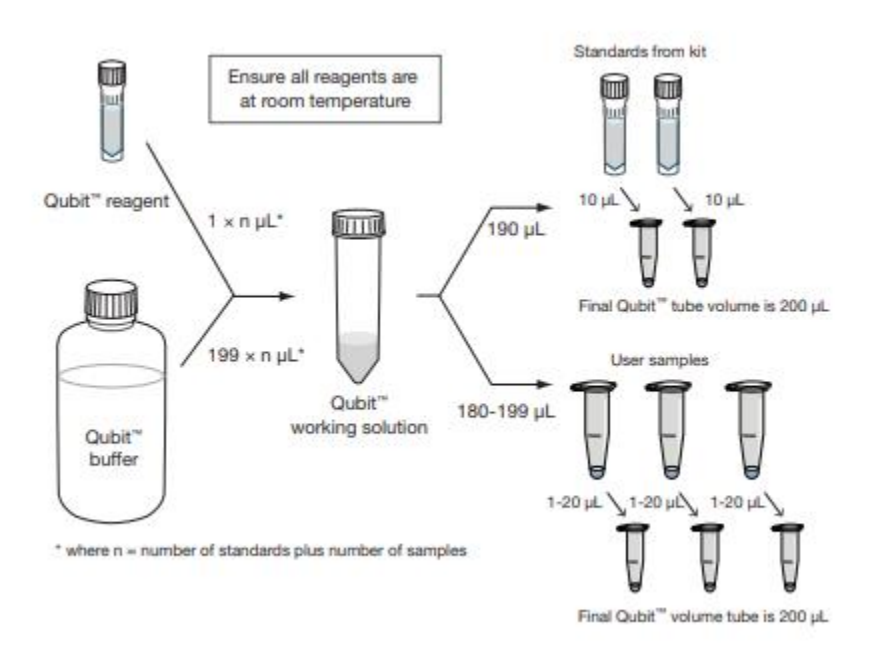
- Process also the control water filter simultaneously with the all samples
- Measure concentration and quality of the DNA extract with nanodrop and QBIT
- Store at -20 °C

3.2 DNA concentration checks using Qubit DNA quantification kit

- Setup two assay tubes for the calibration standards and label Standard 1 and Standard 2
- Prepare the Qubit working solution by diluting the Qubit reagent by 1:200 of working solution for each standard and sample
- Prepare the assay tubes according to the table provided:

	Standard assay tubes	User sample assay tubes
Volume of working solution (from step 2) to add	190 µL	180-199 µL
Volume of standard (from kit) to add	10 µL	-
Volume of user sample to add	-	1-20 µL
Total volume in each assay tube	200 µL	200 µL

- Vortex all tubes for 2-3 seconds
- Incubate the tubes in the Qubit Fluorometer and take the readings. For detailed instructions, please refer to the Qubit.



3.2 DNA concentration checks using Nano drop

- Required materials
  - Kim wipes
  - Sterile gloves
  - Sterile pipette tips (1-10 µL)

- ∇ Clean the nano drop sensor with kim wipes
- ∇ Pipette 1 µL of blank (DNA free or distilled and autoclaved water) onto the sensor
- ∇ Initiate read
- ∇ Record results (Acceptable range (260-280= 1.8-2, 260-230=2.0-2.2)

3.3 PCR DNA amplification 1 CO1 and 18S Metabarcoding Analyses

- Required materials
  - >70% ethanol, 10% bleach for cleaning
  - Sterile gloves
  - 0.2 ml strips of 8 + racks
  - Ice box + ice
  - PCR-grade water
  - Pipettes
  - Filter tips
  - Primers
  - Amplitaq Gold master Mix

- Protocol
  - ∇ Wear gloves at all times. Carefully clean the bench station(s) and pipettes
  - ∇ Prepare calculations and sample map for location of samples in strips
  - ∇ Defrost reagents on ice; prepare mastermix for all samples, accounting for about 10% more volume, for pipetting error. One reaction in a total volume of 15 µl:
    - 0.3 µL (10 uM) of forward and reverse primers
    - 7.5 µL Amplitaq Gold master Mix
    - 5.9 µL PCR-grade water
    - 1 µl DNA extract (5-10 ng, or undiluted)
  - ∇ Mix everything except DNA to prepare the mastermix
  - ∇ Aliquot 14 µL to 0.2 ml eppie strips
  - ∇ Finally add the 1 ul of DNA for each sample
    - 3 reactions for each sample and 1 control

- PCR protocol :
  - 95 °C for 3 min;
  - 35 (or up to 40) cycles
  - COI: 95 °C for 15 s, 50 °C for 30 s, 72 °C for 30 sec;
  - 18S: 95 °C for 45 s, 57 °C for 60 s, 72 °C for 90 sec;
  - Finish with 72 °C for 1 min for both amplicons
- Store products in fridge for short-term (<24 hours) or freezer for longer-term storage

3.4 Quality checks with Gel Electrophoresis

Expect a 313 bp product for COI. And about 260 bp product for 18S.

Prepare the gel for electrophoresis

- Required materials
  - Agarose
  - TAE-buffer
  - SYBRGreen gel dye
  - A microwave
  - Plastic/Glass erlenmeyers
  - Gel casting tray and combs

- Protocol
  - Prepare the gel casting tray with combs
  - Weigh 1,5 – 2 g of agarose and add 100 ml of 1XTAE buffer
  - Heat in the microwave, mix in between, until the agarose is fully dissolved. Be careful that the mix does not over-heat, it can boil over when taking out of the microwave. Be careful it will be very hot when you take it out of the microwave
  - Let the mixture cool shortly, to about 50 C. Add 1 ul of SYBRGreen/GelGreen gel dye
  - Pour the gel slowly into the casting tray, avoid air bubbles on the surface of the gel
  - Allow the gel to solidify (this will take some time)
  - Remove the gel from the casting tray
  - Place in electrophoresis box with enough 1X TBE (or TAE) to cover gel. Gently remove comb.

Run gel electrophoresis

- Required materials
  - Agarose gel

  - Electrophoresis box
  - Power source
  - Loading dye
  - Molecular weight ladder (PCR-100bp or for gDNA-1kb)
  - Gel stain
  - UV-box

- Protocol
  - Add loading buffer to each of your PCR samples (usually 5 µl of PCR sample with 1 µl of loading dye)
  - Carefully load 3 µl of a molecular weight ladder added to 2 µl of into the first lane of the gel
  - Carefully load your samples into the additional wells of the gel
  - Remove gel and run on the gel chamber in 1X TAE buffer at 100V for 1hr
  - Carefully remove gel and visualize on UV trans-illuminator making sure to place the viewing cover to protect your eyes
  - Compare the DNA bands to the DNA ladder to estimate the size of the PCR products.

To check for the concentration of DNA, run a sample with nanodrop and/or Qubit.

3.5 PCR 2 Next Generation Sequencing Library preparation

This section is not covered during the course, but added here for reference.

Table of Indexing primers utilized for the PacMAN

Forward Primer name	**Sequence 3'-5'**	**Index name**
NGS_i5_S502	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTC	S502
NGS_i5_S503	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTC	S503
NGS_i5_S505	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTC	S505
NGS_i5_S506	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTC	S506
NGS_i5_S507	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTC	S507
NGS_i5_S508	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTTCGTCGGCAGCGTC	S508
NGS_i5_S502	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTC	S502

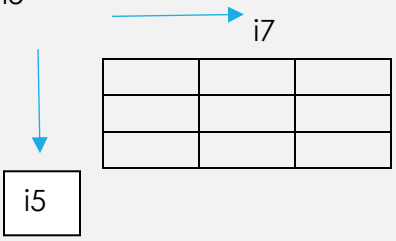
NGS_i5_S510	AATGATACGGCGACCACCGAGATCTACACCGTCTAATTCGTCGGCAGCGTC	S510
NGS_i5_S511	AATGATACGGCGACCACCGAGATCTACACTCTCTCCGTCGTCGGCAGCGTC	S511
NGS_i5_S513	AATGATACGGCGACCACCGAGATCTACACTCGACTAGTCGTCGGCAGCGTC	S513
NGS_i5_S515	AATGATACGGCGACCACCGAGATCTACACTTCTAGCTTCGTCGGCAGCGTC	S515
NGS_i5_S516	AATGATACGGCGACCACCGAGATCTACACCCTAGAGTTCGTCGGCAGCGTC	S516
NGS_i5_S517	AATGATACGGCGACCACCGAGATCTACACGCGTAAGATCGTCGGCAGCGTC	S517
NGS_i5_S518	AATGATACGGCGACCACCGAGATCTACACCTATTAAGTCGTCGGCAGCGTC	S518
NGS_i5_S520	AATGATACGGCGACCACCGAGATCTACACAAGGCTATTCGTCGGCAGCGTC	S520
NGS_i5_S521	AATGATACGGCGACCACCGAGATCTACACGAGCCTTATCGTCGGCAGCGTC	S521
NGS_i5_S522	AATGATACGGCGACCACCGAGATCTACACTTATGCGATCGTCGGCAGCGTC	S522

Reverse Primer name	**Sequence 3'-5'**	**Index name**
NGS_i7_N701	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG	N701
NGS_i7_N702	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGG	N702
NGS_i7_N703	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGG	N703
NGS_i7_N704	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGG	N704
NGS_i7_N705	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGG	N705
NGS_i7_N706	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGG	N706
NGS_i7_N707	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGG	N707

NGS_i7_N710	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGG	N710
NGS_i7_N711	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGG	N711
NGS_i7_N712	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGG	N712
NGS_i7_N714	CAAGCAGAAGACGGCATACGAGATTCATGAGCGTCTCGTGGGCTCGG	N714
NGS_i7_N715	CAAGCAGAAGACGGCATACGAGATCCTGAGATGTCTCGTGGGCTCGG	N715
NGS_i7_N716	CAAGCAGAAGACGGCATACGAGATTAGCGAGTGTCTCGTGGGCTCGG	N716
NGS_i7_N718	CAAGCAGAAGACGGCATACGAGATGTAGCTCCGTCTCGTGGGCTCGG	N718
NGS_i7_N719	CAAGCAGAAGACGGCATACGAGATTACTACGCGTCTCGTGGGCTCGG	N719
NGS_i7_N720	CAAGCAGAAGACGGCATACGAGATAGGCTCCGGTCTCGTGGGCTCGG	N720
NGS_i7_N721	CAAGCAGAAGACGGCATACGAGATGCAGCGTAGTCTCGTGGGCTCGG	N721
NGS_i7_N722	CAAGCAGAAGACGGCATACGAGATCTGCGCATGTCTCGTGGGCTCGG	N722
NGS_i7_N723	CAAGCAGAAGACGGCATACGAGATGAGCGCTAGTCTCGTGGGCTCGG	N723
NGS_i7_N724	CAAGCAGAAGACGGCATACGAGATCGCTCAGTGTCTCGTGGGCTCGG	N724
NGS_i7_N726	CAAGCAGAAGACGGCATACGAGATGTCTTAGGGTCTCGTGGGCTCGG	N726
NGS_i7_N727	CAAGCAGAAGACGGCATACGAGATACTGATCGGTCTCGTGGGCTCGG	N727
NGS_i7_N728	CAAGCAGAAGACGGCATACGAGATTAGCTGCAGTCTCGTGGGCTCGG	N728
NGS_i7_N729	CAAGCAGAAGACGGCATACGAGATGACGTCGAGTCTCGTGGGCTCGG	N729

- **Protocol**
  - Use (cleaned) PCR products from the **first PCR** reaction

- If bands from PCR 1 clear and the secondary band is may exist but only appears lightly, then progression to PCR can continue. If the secondary band is also darker then a clean-up kit will be needed using magnetic beads
- Make map of samples and indexing primers for each 0.2 ml tube before pipetting
- Note i5-Initial binding and i7 end binding



- PCR reaction (20 µl each). Pipette separately in each tube (or using multi pipette, do not add primers into supermix).
- 10 µL µl Amplitaq Gold master Mix
- 4 µL of PCR-grade water
- 0.5 µL (10 nmol/ml) of both F and R primer
- 5 µL of cleaned PCR1 product

- **PCR conditions**
  - initial denaturation step at 95 °C for 10 minutes
  - 15 cycles
  - 95 °C for 0:30, annealing at 55 °C for 0:30, and extension at 72 °C for 1:00.
  - A final extension at 72 °C for 10 minutes.
  - Clean PCR products using QIAquick PCR Purification Kit (Note we used the GeneJET purification kit)
  - Quantify fluorometrically.
  - Pool all samples at an equimolar concentration
  - Dilute samples to the same concentration using PCR-grade water, and combine the same volume of each sample
  - Concentrate sample using the QIAquick PCR purification kit.
  - Quantify DNA concentration of mixture
  - Run 1-5 µL on gel with standard ladder, and image
  - Send for sequencing

- **Equal-molar preparation for freighting of COI and 18S**
  - DNA quantification is conducted using a nanodrop. Minimum cutoff concentrations that are widely accepted by sequencing facilities are 20ug
  - An equal concentration of both COI and 18S products for the same sample ID are combined in the same tube

PacMAN

Example of 96 - well plate setup for library construction with dual indexing primers:

	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S502	S502 + N701	S502 + N702	S502 + N703	S502 + N704	S502 + N705	S502 + N706	S502 + N707	S502 + N710	S502 + N711	S502 + N712	S502 + N714	S502 + N715
S503	S503 + N701	S503 + N702	S503 + N703	S503 + N704	S503 + N705	S503 + N706	S503 + N707	S503 + N710	S503 + N711	S503 + N712	S503 + N714	S503 + N715
S505	S505 + N701	S505 + N702	S505 + N703	S505 + N704	S505 + N705	S505 + N706	S505 + N707	S505 + N710	S505 + N711	S505 + N712	S505 + N714	S505 + N715
S506	S506 + N701	S506 + N702	S506 + N703	S506 + N704	S506 + N705	S506 + N706	S506 + N707	S506 + N710	S506 + N711	S506 + N712	S506 + N714	S506 + N715
S507	S507 + N701	S507 + N702	S507 + N703	S507 + N704	S507 + N705	S507 + N706	S507 + N707	S507 + N710	S507 + N711	S507 + N712	S507 + N714	S507 + N715
S508	S508 + N701	S508 + N702	S508 + N703	S508 + N704	S508 + N705	S508 + N706	S508 + N707	S508 + N710	S508 + N711	S508 + N712	S508 + N714	S508 + N715
S510	S510 + N701	S510 + N702	S510 + N703	S510 + N704	S510 + N705	S510 + N706	S510 + N707	S510 + N710	S510 + N711	S510 + N712	S510 + N714	S510 + N715
S511	S511 + N701	S511 + N702	S511 + N703	S511 + N704	S511 + N705	S511 + N706	S511 + N707	S511 + N710	S511 + N711	S511 + N712	S511 + N714	S511 + N715

3.6 qPCR analyses

For species-specific detections, existing assays can be used if there are literature available. New assays can be developed for each of the chosen target risk species should there be an unavailability of existing assays to ensure that the utilized primers do not have unspecific amplification of closely related local species. Some existing assays for the species in the target list are reviewed in table 3. These primers have been tested for qPCR functionality and have been found to be species-specific, and do not amplify a range of closely related species from the region of testing (not Fiji).

Table showing already developed primers for species targeted detection for PacMAN

Target Species	**Primer name**	**Forward sequence**	**T (C)**	**Reverse sequence**	**T (C)**	**Length**	**Probe sequence**	**Reference**
Eriochier sinensis	Erisin_cytb_F02/R02	ACCCCTCCTCATATCCAACCA	62.7	AAGAATGGCCACTGAAGCGG	64.7	114	FAM-TTTGCTTACGCTATTTTACGATCAATTCCT-BHQ1	Andersen et al. 2018
Rhithropanopeus harrisii	Rhihar_cytb_F03/R03	GTCAACCTGGTACTCTCATTGGT	63	ACGAGGAAATGCTATATCAGGGG	63	164	FAM-TGTTGTAGTAACAGCTCACGCCTTTGT-BHQ1	Andersen et al. 2018
Mytilopsis sallei	MytF, MytR	GYTAGTTCCRATGATGTTAGCTG		ACCTATTGAAACAGGCAACACTC			CCTCGGCTTAATAATGTTAGT	Bott et al. 2012
*Perna perna**	Fw A, Rev A	CTTAGTGGCATTAATTCGDAATCC	59.2	CAAAGTACCAATATCTTTATGATTRGTWGA	57.5	281	AACCATCGACTCAATTAA (lagging DNA strand)	Dias et al. 2013
*Perna viridis**	Fw A, Rev A	CTTAGTGGCATTAATTCGDAATCC	59.2	CAAAGTACCAATATCTTTATGATTRGTWGA	57.5	281	ACTCAAACAACAAAGTAAAC (lagging DNA strand)	Dias et al. 2013
Didemnum perlucidum	Dper new F/R	AGCTCCTGATATAGCATTTCCTCGTTTAAA	63.3	AGATATTCCTGCTAAATGTAATGAAAAAATAGCTA	61.2		TAGCTCATTCAAATAGGGCAGTA	Simpson et al. 2017

Mytella strigata	CO1 mytellaFf/r	GGGTTAATAGGAAGAAGGTTGAGA	50 used	ACAACCACCGATACATAAAGG	50 used	196	*Not developed*	Yip et al. 2021
Hemigrapsus sanguineus	Hemsan_COI_F/R01	CCTGGGCCGGTATAGTAGGT	60.2	GGGGCTCCGAGTATAAGTGG	59.3	136	CGAGCAGAATTAAGACAACCAGGAAGC	Knudsen et al. 2020

For this course we will be running one target species analysis for Hemigrapsus sanguineus (the Japanese shore crab). You will analyse:

- 3 environmental samples in triplicate
- One non-template control (NTC; PCR-water)
- 5 dilutions of the positive control DNA (1 ng/µl)

- Required materials:
  - DNA extract of unknown samples - 10 ng to 100 ng gDNA
  - Target species DNA diluted for a standard curve
  - Forward and reverse primers diluted to working concentration (10µM working stocks are sufficient for most assays)
  - Fluorescent Probe (sometimes referred to as TaqMAN probes)
  - Sterile filter pipette tips
  - Sterile 1.5 mL Eppendorf tubes
  - PCR tubes/strips
  - PCR grade water

- **Protocol**
  - Place all reaction components on ice.
  - Calculate the amount needed to prepare enough master mix to run all samples in triplicate, and a standard curve with 5 dilutions.
  - Be sure to include a no template Negative Controls (NTC)
  - Calculate amount of reagents to mix. Add 10% volume to allow for pipetting error
  - Mix well, avoiding bubbles.
- Mastermix (for qPCR ready mixes)
  - For each reaction calculate the following reagents and combine:
    - 5 µl of IDT Primetime Master Mix (2X qPCR mix)
    - 0.5 µl 10 µM concentration forward primer (0.5 µM final concentration)
    - 0.5 µl 10 µM concentration reverse primer (0.5 µM final concentration)
    - 0.5ul x 5 µM Probe (0.25 µM final concentration)
    - 1.5 µl PCR water

        - Account for about 10% extra for pipetting error
- Setup reactions:
    - Carefully aliquot 8 µL of template master mix into each qPCR tube or plate well.
    - For NTC reactions, add 2 µL of water to the empty reaction tube, close well
    - For experimental reactions, add 2 µL of DNA solution to the empty reaction tubes.
    - Mix reactions well and spin if needed.
    - Cap tubes or seal the PCR plate and label. Make sure the labelling does not obscure instrument excitation/detection light path.)

- Run samples with the following cycling parameters:
    - Initial denaturation 95 °C for 60 seconds
    - 40 cycles:
        - Denaturation 95 °C for 30 seconds
        - Annealing and extension 60 °C for 60 seconds
        - Fluorescence read after each annealing phase

Calculate your DNA concentration based on the known concentrations in your standard curve.

Topic 4: Data management and bioinformatics

During the course you will run through an example workflow of the PacMAN bioinformatics pipeline (available at: https://github.com/iobis/PacMAN-pipeline) and examples of data analysis in R.

Tutorials for the bioinformatics and data analysis have been developed and will be run with local computers in Fiji.

All information of the tutorials can be found

1. Bioinformatics pipeline tutorial:

   https://github.com/iobis/pacman-pipeline-training/blob/master/tutorial.md

2. Data analysis tutorial:

   https://iobis.github.io/pacman-pipeline-training/rey_analysis.html

PacMAN

PacMAN DNA extraction (all samples)

Pre-conditioning (day prior to extraction day)

Date:_____(YYYY/MM/DD) Time: _____

1. (✓) Soak glass beads in 2% bleach for 60 min: _____ 2. Rinse at least 3x (milliQ water) & dry: _____

Extraction Day

1. (✓) Add 0.25 g beads into each 2.0 mL screw cap tube:_____2. (✓) Autoclave tubes with other materials (30 mins): _____

3. (✓) Bench Sterilized (> 70 % EtOH & 2 % Bleach):_____4. (✓) Transfer sample to bead tubes (100 mg /200 µL): _____

5. (✓) Add 720 µL Buffer ATL (Qiagen): _____ 6. (✓) Perform bead beating (max. speed for 45 secs): _____

7. (✓) Incubation at 56 °C for 30 minutes: _____ 8. (✓) Repeat steps 6 & 7: _____

9. (✓) Add 80 µL Proteinase K: _____ 10. (✓) Incubate at 56 °C for 2 hrs. - Overnight: _____

After Incubation

11. (✓) Vortex tubes for 15 sec: _____ 12. (✓) Centrifuge for 1 min at 4,000 x g: _____

13. (✓) Transfer 650 µL supernatant (avoid glass beads) to new tube: _____ 14. (✓) Spin at 13, 000 x g for 1 min: _____

15. (✓) Transfer 600 µL of supernatant (avoid glass beads) to new tube: _____ 16. (✓) Add 600 µL Buffer AL to tube: _____

17. (✓) Mix thoroughly by vortexing: _____ 18. (✓) Add 600 µL 100 % EtOH and vortex: ____

It is essential that the sample, Buffer AL and ethanol are mixed immediately and thoroughly by vortexing. A white precipitate may form on addition of Buffer AL and EtOH. This precipitate does not interfere with the DNeasy procedure. Some tissue types may form a gelatinous lysate after addition. In this case, vigorously shaking and vortexing the preparation is recommended.

19. (✓) Pipette 500 µL OF lysate to spin column: ____ 20. (✓) Centrifuge (8000 rpm, 1 min): _____

21. (✓) Discard flow-through: _____

22. (✓) Repeat steps 19 -21 until entire volume of lysate passes through the column: _____

23. (✓) Change collection tube: _____ 23. (✓) Add 500 µL Buffer AW1: _____

24. (✓) Centrifuge (8000 rpm, 1 min): _____ 25. (✓) Discard flow-through: _____

26. (✓) Repeat steps 23 & 24: _____ 27. (✓) Change collection tube: _____

28. (✓) Add 500 µL Buffer AW2: _____ 29a. (✓) Centrifuge (14000 rpm, 3 min): ___

29b. (✓) Discard flow-through: _____ 30. (✓) Repeat steps 28 & 29: _____

It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000rpm).

31. (✓) Place DNeasy spin column in a clean, pre-labelled tube:_____32. (✓) Add 50 µL AE Buffer into tube: _____

33. (✓) Incubate at room temperature (1 min.): _____ 34. (✓) Centrifuge (8000 rpm, 1 min.): _____

35. (✓) Keep 1st eluted DNA: _____ 36. (✓) Repeat steps 31-35 for 2nd eluted DNA: _

37. Storage location of DNA Samples in -20 °C freezer: _____

Water processing datasheet

PacMAN Water Sample Processing Data sheet

1. (✓) Bench Sterilized (> 70 % EtOH & 2 % Bleach): _____ 2. (✓) Sterilized filtration system & materials used: ____

Sample Information:

3. Date: _____ (YYYY/MM/DD) 4. Time: _____

5. (✓) 1L bottle of deionized water open: ____ ***(for the duration of all water filtration) only when beginning***

Replicate 1 **ID:** _____

6. Type of filter paper: _____ 7. Volume of Sample: _____ mL

8. (✓) Condition filter (DI water): _____ 9. (✓) Filter water: _____

10. No. of Filter paper used: _____ 11. If more than 1, volume filtered in 1st filter paper: ____ mL

12. (✓) Place filter paper in sterilized tube (Use tweezer): _____ 13. (✓) Discard filtered water &Label tube: ____

Replicate 2 **ID:** _____

14. Type of filter paper: _____ 15. Volume of Sample: _____ mL

16. (✓) Condition filter (DI water): _____ 17. (✓) Filter water: _____

18. No. of Filter paper used: _____ 19. If more than 1, volume filtered in 1st filter paper: ____ mL

20. (✓) Place filter paper in sterilized tube (Use tweezer): _____ 21. (✓) Discard filtered water &Label tube: ____

Replicate 3 **ID:** _____

22. Type of filter paper: _____ 23. Volume of Sample: _____ mL

24. (✓) Condition filter (DI water): _____ 25. (✓) Filter water: _____

26. No. of Filter paper used: _____ 27. If more than 1, volume filtered in 1st filter paper: ____ mL

28. (✓) Place filter paper in sterilized tube (Use tweezer): _____ 29. (✓) Discard filtered water &Label tube: ____

(Skip to step 33. When filtering last sample of the day)

30. (✓) Clean filtration cup and holder with bleach and deionized water: _____

31. (✓) Change gloves for next site samples: _____ 32. Start new Data sheet for next site

Control: **ID:** _____

33. (✓) Bench Sterilized (> 70 % EtOH & 2 % Bleach): _____ 34. (✓) Sterilized filtration system: _____

35. Type of filter paper: _____ 36. Volume of Sample: _____ mL

37. (✓) Place filter paper in sterilized tube: _____ 38. (✓) Label on tube: ____

39. (✓) Place all labelled samples in the freezer: _____

Plankton sample processing sheet

PacMAN

PacMAN Plankton Sample Processing Data sheet

1. .Date: _____ (YYYY/MM/DD) 2. Time: _____

3. (✓) Bench Sterilized (> 70 % EtOH & 2 % Bleach): _____ 4. (✓) Sterilized falcon tubes and holder: ____

Sample Information **Site:** _____

Replicate 1 **ID:** _____

5. Volume of Sample: _____ mL

6. (✓) Pour sample into falcon tubes: _____ 7. (✓) Centrifuge to form pellets: _____

8. (✓) Decant samples and combine: _____ 9. (✓) Label tube containing samples: _____

Replicate 2 **ID:** _____

10. Volume of Sample: _____ mL

11. (✓) Pour sample into falcon tubes: _____ 12. (✓) Centrifuge to form pellets: _____

13. (✓) Decant samples and combine: _____ 14. (✓) Label tube containing samples: _____

Replicate 3 **ID:** _____

15. Volume of Sample: _____ mL

16. (✓) Pour sample into falcon tubes: _____ 17. (✓) Centrifuge to form pellets: _____

18. (✓) Decant samples and combine: _____ 19. (✓) Label tube containing samples: _____

(Sterilize bench and change gloves for next site)

Sample Information **Site:** _____

Replicate 1 **ID:** _____

5. Volume of Sample: _____ mL

6. (✓) Pour sample into falcon tubes: _____ 7. (✓) Centrifuge to form pellets: ____

8. (✓) Decant samples and combine: _____ 9. (✓) Label tube containing samples: ____

Replicate 2 **ID:** _____

10. Volume of Sample: _____ mL

11. (✓) Pour sample into falcon tubes: _____ 12. (✓) Centrifuge to form pellets: ____

13. (✓) Decant samples and combine: _____ 14. (✓) Label tube containing samples: ____

Replicate 3 **ID:** _____

15. Volume of Sample: _____ mL

16. (✓) Pour sample into falcon tubes: _____ 17. (✓) Centrifuge to form pellets: ____

18. (✓) Decant samples and combine: _____ 19. (✓) Label tube containing samples: ____

20. (✓) Place all samples in -20 freezer: _____